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Detailed Description of the Invention

In order that the invention herein described may be more fully understood, the following detailed description is set forth.

The present invention relates to novel antibodies that are highly specific for carcinoma cells. More particularly, the antibodies react with a range of carcinomas such as breast, lung, ovary and colon carcinomas, while showing none or limited reactivity with normal human tissues or other types of tumors such as sarcomas or lymphomas.

The BR96 antibodies can be used to isolate and characterize the antigen to which they bind. Thus, the BR96 antibodies can be used as a probe to identify and characterize the epitope recognized and to further define the cell membrane antigen with which they react [see, e.g., Nudelman et al., "Characterization of Human Melanoma-Associated Ganglioside Antigen Defined By A Monoclonal Antibody, 4.2", J. Biol. Chem., 257 (1)12752-56 (1982) and Hakomori, "Tumor Associated Carbohydrate Antigens", Ann. Rev. Immunol., 2:103-26 (1984)].

Results of preliminary epitope screens conducted on monoclonal antibody BR96 have indicated that the antigen on the carcinoma cells to which BR96 antibody binds is a fucosylated variant of the Lewis Y antigen. The Lewis Y (Le^y) antigen has been described by Abe et al., J. Biol. Chem. 258:8934 (1983); Lloyd et al., Immunogenetics 17:537 (1983); Brown et al., Biosci. Rep. 3:163 (1983); Hellstrom et al., Cancer Res. 46:3917 (1986). Fucosylated Lewis Y antigen has been described by Abe et al., Cancer Res. 46:2639-2644 (1986).

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The monoclonal antibody of the invention can be produced using well-established hybridoma techniques first introduced by Kohler and Milstein [see, Kohler and Milstein, "Continuous Cultures Of Fused Cells Secreting Antibody Of Pre-Defined Specificity", Nature, 256:495-97 (1975). See, also, Brown et al., "Structural Characterization Of Human Melanoma-Associated Antigen p97 with Monoclonal Antibodies", J. Immunol., 127 (2):539-46 (1981)]; Brown et al., "Protein Antigens Of Normal And Malignant Human Cells Identified By Immunoprecipitation With Monoclonal Antibodies", J. Biol. Chem., 255:4980-83 (1980); Yeh et al., "Cell Surface Antigens Of Human Melanoma Identified By Monoclonal Antibody", Proc. Natl. Acad. Sci. USA, 76(6):297-31 (1979); and Yeh et al., "A Cell-Surface Antigen Which is Present In the Ganglioside Fraction And Shared By Human Melanomas", Int. J. Cancer, 29:269-75 (1982).]

These techniques involve the injection of an immunogen (e.g., cells or cellular extracts carrying the antigen or purified antigen) into an animal (e.g., a mouse) so as to elicit a desired immune response (i.e., antibodies) in that animal. After a sufficient time, antibody-producing lymphocytes are obtained from the animal either from the spleen, lymph nodes or peripheral blood. Preferably, the lymphocytes are obtained from the spleen. The splenic lymphocytes are then fused with a myeloma cell line, usually in the presence of a fusing agent such as polyethylene glycol (PEG). Any of a number of myeloma cell lines may be used as a fusion partner according to standard techniques; for example, the P3-NS1/1A$_g$4-1, P3-x63-Ag8.653 or Sp2/0 Ag14 myeloma lines. These myeloma lines are available from the American Type Culture Collection, ("ATCC") in Rockville, Maryland.

The resulting cells, which include the desired hybridomas, are then grown in a selective medium, such as

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HAT medium, in which unfused parental myeloma or
lymphocyte cells eventually die. Only the hybridoma
cells survive and can be grown under limiting conditions
to obtain isolated clones. The supernatants of the
hybridomas are screened for the presence of antibody of
that desired specificity, e.g., by immunoassay techniques
using the antigen that had been used for immunization.
Positive clones can then be subcloned under limiting
dilution conditions and the monoclonal antibody produced
can be isolated. Hybridomas produced according to these
methods can be propagated in vitro or in vivo (in ascites
fluid) using techniques known in the art [see, generally,
Fink et al., supra at page 123, Fig. 6-11]. Commonly
used methods for purifying monoclonal antibodies include
ammonium sulfate precipitation, ion exchange
chromatography, and affinity chromatography [see, e.g.,
Zola et al., "Techniques For The Production And
Characterization Of Monoclonal Hybridoma Antibodies", in
Monoclonal Hybridoma Antibodies: Techniques And
Applications, Hurell (ed.), pp. 51-52 (CRC Press 1982)].

    According to a preferred embodiment, a
monoclonal antibody of this invention, designated BR96,
was produced via the hybridoma techniques described
hereinbelow using a breast cancer cell line 3396 as the
immunogen. The BR96 hybridoma, prepared as described
hereinbelow and producing the BR96 antibody, was
deposited on February 22, 1989 with the ATCC, and has
there been identified as follows:

        BR96 ATCC Accession No.: HB 10036

    The BR96 antibody is of the IgG3 subclass. The
antibody displays a high specificity for carcinoma cells
of different organ types, for example, tumors of the
breast, lung, colon and ovary as well as cultured cell
lines established from various breast, lung and colon

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carcinomas. Furthermore, the BR95 antibody shows no binding to other types of tumor cells such as the T-cell lymphoma cells lines, CEM and MOLT-4, the B cell lymphoma cell line P3HR-1 or melanoma cells lines. The BR96 antibody is able to be internalized in antigen-positive tumor cells, is toxic on antigen-positive tumor cells, mediates ADCC and CDC activity, and surprisingly, is cytotoxic alone, i.e. in unmodified form. The BR96 antibodies appear to recognize a Le^y antigen.

According to another embodiment, F(ab')$_2$ fragments of the BR96 monoclonal antibody were produced by pepsin digestion of purified BR96 [Nisonoff et al., "The Antibody Molecule",

Academic Press, New York (1975)], as described hereinbelow. The binding of the F(ab')$_2$ fragments to tumor (3396) and MCF7 cells was shown to be comparable to the binding of the whole BR96 monoclonal antibody.

In another preferred embodiment, the chimeric (murine/human) antibody of the invention was produced using a two-step homologous recombination procedure as described by Fell et al., in Proc. Natl. Acad. Sci. USA 86:8507-8511 (1989) and in co-pending patent application U.S. Serial Number 243,873, filed September 14, 1988, and Serial No. 468,035, filed June 22, 1990, assigned to the same assignee as the present application; the disclosures of all of these documents are incorporated in their entirety by reference herein. This two-step protocol involves use of a target vector encoding human IgGgamma1 heavy chain to transfect a mouse hybridoma cell line expressing murine BR96 monoclonal antibody (hybridoma ATCC No. HB 10036) to produce a hybridoma expressing a BR96 chimeric antibody containing human IgGgamma1 heavy chain. This hybridoma is then transfected with a target vector containing DNA encoding human kappa (K) light

chain to produce a murine hybridoma expressing a BR96
chimeric antibody containing human IgGgamma1 heavy chain
and human K light chain.   The target vectors used to
transfect the hybridomas are the pHgamma1HC-DD$_4$ vector
digested with Xbal enzyme (Oncogen, Seattle, WA) and the
HindIII digested pSV$_2$gpt/C$_K$ vector (Oncogen, Seattle, WA).

The chimeric BR96 hybridoma, identified herein
as ChiBR96, prepared as described hereinbelow and
producing the
chimeric human/murine BR96 antibody, was deposited on May
23,
1990, with the ATCC, and has there been identified as
follows:

ChiBR96 ATCC Accession No.: HB 10460

Once the hybridoma that expresses the chimeric
antibody is identified, the hybridoma is cultured and the
desired chimeric molecules are isolated from the cell
culture supernatant using techniques well known in the
art for isolating monoclonal antibodies.

The term "BR96 antibody" as used herein
includes whole, intact polyclonal and monoclonal antibody
materials such as the murine BR96 monoclonal antibody
produced by hybridoma ATCC No. HB 10036, and chimeric
antibody molecules such as chimeric BR96 antibody
produced by hybridoma ATCC No. 10460. The BR96 antibody
described above includes any fragments thereof containing
the active antigen-binding region of the antibody such as
Fab, F(ab')$_2$ and Fv fragments,using techniques well
established in the art [see, e.g., Rouseaux et al.,
"Optimal Conditions For The Preparation of Proteolytic
Fragments From Monoclonal IgG of Different Rat IgG
Subclasses", in Methods Enzymol., 121:663-69 (Academic

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Press 1986)]. The BR96 antibody of the invention also
includes fusion proteins.

In addition, the BR96 antibody of this
invention does not display any immunohistologically
detectable binding to normal human tissues from major
organs, such as kidney, spleen, liver, skin, lung,
breast, colon, brain, thyroid, heart, lymph nodes or
ovary. Nor does the antibody react with peripheral blood
leukocytes. BR96 antibody displays limited binding to
some cells in the tonsils and testes, and binds to acinar
cells in the pancreas, and to epithelial cells in the
stomach and esophagus. Thus, the BR96 antibody is
superior to most known antitumor antibodies in the high
degree of specificity for tumor cells as compared to
normal cells [see, e.g., Hellstrom et al., "Immunological
Approaches To Tumor Therapy: Monoclonal Antibodies, Tumor
Vaccines, And Anti-Idiotypes", in Covalently Modified
Antigens And Antibodies In Diagnosis And Therapy,
Quash/Rodwell (eds.), pp. 1-39 (Marcell Dekker, Inc.,
1989) and Bagshawe, "Tumour Markers - Where Do We Go From
Here", Br. J. Cancer, 48:167-75 (1983)].

In addition, the present invention encompasses
antibodies that are capable of binding to the same
antigenic determinant as the BR96 antibodies and
competing with the antibodies for binding at that site.
These include antibodies having the same antigenic
specificity as the BR96 antibodies but differing in
species origin, isotype, binding affinity or biological
functions (e.g., cytotoxicity). For example, class,
isotype and other variants of the antibodies of the
invention having the antigen-binding region of the BR96
antibody can be constructed using recombinant
class-switching and fusion techniques known in the art
[see, e.g., Thammana et al., "Immunoglobulin Heavy Chain
Class Switch From IgM to IgG In A Hybridoma", Eur. J.

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Immunol., 13:614 (1983); Spira et al., "The Identification Of Monoclonal Class Switch Variants By Subselection And ELISA Assay", J. Immunol. Meth., 74:307-15 (1984); Neuberger et al., "Recombinant Antibodies Possessing Novel Effector Functions", Nature, 312: 604-608 (1984); and Oi et al., "Chimeric Antibodies", Biotechniques, 4 (3):214-21 (1986)]. Thus, other chimeric antibodies or other recombinant antibodies (e.g., fusion proteins wherein the antibody is combined with a second protein such as a lymphokine or a tumor inhibitory growth factor) having the same binding specificity as the BR96 antibodies fall within the scope of this invention.

Also included within the scope of the invention are anti-idiotypic antibodies to the BR96 antibody of the invention. These anti-idiotypic antibodies can be produced using the BR96 antibody and the fragments thereof as immunogen and are useful for diagnostic purposes in detecting humoral response to tumors and in therapeutic applications, e.g., in a vaccine, to induce an anti-tumor response in patients [see, e.g., Nepom et al., "Anti-Idiotypic Antibodies And The Induction Of Specific Tumor Immunity", in Cancer And Metastasis Reviews, 6:487-501 (1987)].

The BR96 antibody of the invention is also useful for diagnostic applications, both in vitro and in vivo, for the detection of human carcinomas that possess the antigen for which the antibodies are specific. In vitro diagnostic methods include immunohistological detection of tumor cells (e.g., on human tissue, cells or excised tumor specimens) or serologic detection of tumor-associated antigens (e.g., in blood samples or other biological fluids).

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Immunohistochemical techniques involve staining
a biological specimen such as a tissue specimen with the
BR96 antibody of the invention and then detecting the
presence on the specimen of the antibody complexed to its
antigen. The formation of such antibody-antigen
complexes with the specimen indicates the presence of
carcinoma cells in the tissue. Detection of the antibody
on the specimen can be accomplished using techniques
known in the art such as immunoenzymatic techniques,
e.g., the immunoperoxidase staining technique or the
avidin-biotin (ABC) technique, or immunofluorescence
techniques [see, e.g., Ciocca et al.,
"Immunohistochemical Techniques Using Monoclonal
Antibodies", Meth. Enzymol., 121:562-79 (1986);
Hellstrom et al., "Monoclonal Mouse Antibodies Raised
Against Human Lung Carcinoma", Cancer Research,
46:3917-23 (1986); and Kimball (ed.), Introduction To
Immunology (2nd Ed), pp. 113-117 (Macmillan Pub. Co.
1986)]. For example, immunoperoxidase staining was used
as described in Example 2, infra, to demonstrate the
reactivity of the BR96 antibody with lung, breast, colon,
and ovary carcinomas and the low reactivity of the
antibody with normal human tissue specimens.

Serologic diagnostic techniques involve the
detection and quantitation of tumor-associated antigens
that have been secreted or "shed" into the serum or other
biological fluids of patients thought to be suffering
from carcinoma. Such antigens can be detected in the
body fluids using techniques known in the art such as
radioimmunoassays (RIA) or enzyme-linked immunosorbent
assays (ELISA) wherein an antibody reactive with the
"shed" antigen is used to detect the presence of the
antigen in a fluid sample [see, e.g., Uotila et al.,
"Two-Site Sandwich ELISA With Monoclonal Antibodies To
Human AFP", J. Immunol. Methods, 42:11 (1981) and Allum
et al., supra at pp. 48-51]. These assays, using the BR96

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antibodies disclosed herein, can therefore be used for the detection in biological fluids of the glycolipid antigen with which the BR96 antibodies react and thus the detection of human carcinoma in patients. Thus, it is apparent from the foregoing that the BR96 antibodies of the invention can be used in most assays involving antigen-antibody reactions. These assays include, but are not limited to, standard RIA techniques, both liquid and solid phase, as well as ELISA assays, immunofluorescence techniques, and other immunocytochemical assays [see, e.g., Sikora et al. (eds), Monoclonal Antibodies, pp. 32-52 (Blackwell Scientific Publications 1984)].

The invention also encompasses diagnostic kits for carrying out the assays described above. In one embodiment, the diagnostic kit comprises the BR96 monoclonal antibody, fragments thereof, fusion proteins or chimeric antibody of the invention, and a conjugate comprising a specific binding partner for the BR96 antibody and a label capable of producing a detectable signal. The reagents can also include ancillary agents such as buffering agents and protein stabilizing agents· (e.g., polysaccharides). The diagnostic kit can further comprise, where necessary, other components of the signal-producing system including agents for reducing background interference, control reagents or an apparatus or container for conducting the test. In another embodiment, the diagnostic kit comprises a conjugate of the BR96 antibodies of the invention and a label capable of producing a detectable signal. Ancillary agents as mentioned above can also be present.

The BR96 antibody of the invention is also useful for in vivo diagnostic applications for the detection of human carcinomas. One such approach involves the detection of tumors in vivo by tumor imaging

techniques. According to this approach, the BR96 antibody is labeled with an appropriate imaging reagent that produces a detectable signal. Examples of imaging reagents that can be used include, but are not limited to, radiolabels such as ^{131}I, ^{111}In, ^{123}I, ^{99m}Tc, ^{32}P, ^{125}I, ^{3}H, and ^{14}C, fluorescent labels such as fluorescein and rhodamine, and chemiluminescers such as luciferin. The antibody can be labeled with such reagents using techniques known in the art. For example, see Wensel and Meares, Radioimmunoimaging And Radioimmunotherapy, Esevier, New York (1983) for techniques relating to the radiolabeling of antibodies [see also, Colcher et al., "Use Of Monoclonal Antibodies As Radiopharmaceuticals For The Localization Of Human Carcinoma Xenografts In Athymic Mice", Meth. Enzymol., 121:802-16 (1986)].

In the case of radiolabeled antibody, the antibody is administered to the patient, localizes to the tumor bearing the antigen with which the antibody reacts, and is detected or "imaged" in vivo using known techniques such as radionuclear scanning using, e.g., a gamma camera or emission tomography [see, e.g., Bradwell et al., "Developments In Antibody Imaging", in Monoclonal Antibodies For Cancer Detection And Therapy, Baldwin et al. (eds.), pp. 65-85 (Academic Press 1985)]. The antibody is administered to the patient in a pharmaceutically acceptable carrier such as water, saline, Ringer's solution, Hank's solution or nonaqueous carriers such as fixed oils. The carrier may also contain substances that enhance isotonicity and chemical stability of the antibody such as buffers or preservatives. The antibody formulation is administered, for example, intravenously, at a dosage sufficient to provide enough gamma emission to allow visualization of the tumor target site. Sufficient time should be allowed between administration of the antibody and detection to allow for localization to the tumor target. For a

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general discussion of tumor imaging, see Allum et al.,
supra at pp. 51-55.

The properties of the BR96 antibody: a) very
high specificity for tumor cells; b) internalization; c)
toxicity to antigen-positive tumor cells alone, i.e. in
unmodified form, when used at appropriate concentrations;
and d) CDC and ADCC activity, suggest a number of in vivo
therapeutic applications. First, the BR96 antibody can
be used alone to target and kill tumor cells in vivo.
The antibody can also be used in conjunction with an
appropriate therapeutic agent to treat human carcinoma.
For example, the antibody can be used in combination with
standard or conventional treatment methods such as
chemotherapy, radiation therapy or can be conjugated or
linked to a therapeutic drug, or toxin, as well as to a
lymphokine or a tumor-inhibitory growth factor, for
delivery of the therapeutic agent to the site of the
carcinoma. Techniques for conjugating such therapeutic
agents to antibodies are well known [see, e.g., Arnon et
al., "Monoclonal Antibodies For Immunotargeting Of Drugs
In Cancer Therapy", in Monoclonal Antibodies And Cancer
Therapy, Reisfeld et al. (eds.), pp. 243-56 (Alan R.
Liss, Inc. 1985); Hellstrom et al., "Antibodies For Drug
Delivery", in Controlled Drug Delivery (2nd Ed.),
Robinson et al. (eds.), pp. 623-53 (Marcel Dekker, Inc.
1987); Thorpe, "Antibody Carriers Of Cytotoxic Agents In
Cancer Therapy: A Review", in Monoclonal Antibodies '84:
Biological And Clinical Applications, Pinchera et al.
(eds.), pp. 475-506 (1985); and Thorpe et al., "The
Preparation And Cytotoxic Properties Of Antibody-Toxin
Conjugates", Immunol. Rev., 62:119-58 (1982)]. The BR96
antibody of the invention is particularly suited for use
in a therapeutic conjugate because it is readily
internalized within the carcinoma cells to which it binds
and thus can deliver the therapeutic agent to
intracellular sites of action.

Alternatively, the BR96 antibody can be coupled to high-energy radiation, e.g., a radioisotope such as ^{131}I, which, when localized at the tumor site, results in a killing of several cell diameters [see, e.g., Order, "Analysis, Results, And Future Prospective Of The Therapeutic Use Of Radiolabeled Antibody In Cancer Therapy", in Monoclonal Antibodies For Cancer Detection And Therapy, Baldwin et al. (eds.), pp. 303-16 (Academic Press 1985)]. According to yet another embodiment, the BR96 antibody can be conjugated to a second antibody to form an antibody heteroconjugate for the treatment of tumor cells as described by Segal in United States Patent 4,676,980.

Still other therapeutic applications for the BR96 antibody of the invention include conjugation or linkage, e.g., by recombinant DNA techniques, to an enzyme capable of converting a prodrug into a cytotoxic drug and the use of that antibody-enzyme conjugate in combination with the prodrug to convert the prodrug to a cytotoxic agent at the tumor site [see, e.g., Senter et al., "Anti-Tumor Effects Of Antibody-alkaline Phosphatase", Proc. Natl. Acad. Sci. USA, 85:4842-46 (1988); "Enhancement of the in vitro and in vivo Antitumor Activities of Phosphorylated Mitomycin C and Etoposide Derivatives by Monoclonal ANtiobdy-Alkaline Phosphatase Conjugates", Cancer Research 49:5789-5792 (1989); and Senter, "Activation of Prodrugs by Antibody-Enzyme Conjugates: A New Approach to Cancer Therapy, FASEB J. 4:188-193 (1990)]. Still another therapeutic use for the BR96 antibody involves use, either in the presence of complement or as part of an antibody-drug or antibody-toxin conjugate, to remove tumor cells from the bone marrow of cancer patients. According to this approach, autologous bone marrow may be purged ex vivo by treatment with the antibody and the marrow infused back into the patient [see, e.g., Ramsay et al., "Bone Marrow

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Purging Using Monoclonal Antibodies", J. Clin. Immunol.,
8(2):81-88 (1988)].

        Furthermore, chimeric or other recombinant BR96
antibodies of the invention, as described earlier, may be
used therapeutically. For example, a fusion protein
comprising at least the antigen-binding region of the
BR96 antibody joined to at least a functionally active
portion of a second protein having anti-tumor activity,
e.g., a lymphokine or oncostatin, may be used to treat
human carcinoma in vivo. Furthermore, recombinant
techniques known in the art can be used to construct
bispecific antibodies wherein one of the binding
specificities of the antibody is that of BR96 [see, e.g.
United States Patent 4,474,893].

        Finally, anti-idiotypic antibodies of the BR96
antibody may be used therapeutically in active tumor
immunization and tumor therapy [see, e.g., Hellstrom et
al., "Immunological Approaches To Tumor Therapy:
Monoclonal Antibodies, Tumor Vaccines, And
Anti-Idiotypes", in Covalently Modified Antigens And
Antibodies In Diagnosis And Therapy, supra at pp.
35-41].

        It is apparent therefore that the present
invention encompasses pharmaceutical compositions,
combinations and methods for treating human carcinomas.
For example, the invention includes pharmaceutical
compositions for use in the treatment of human carcinomas
comprising a pharmaceutically effective amount of a BR96
antibody and a pharmaceutically acceptable carrier. The
compositions may contain the BR96 antibody either
unmodified, conjugated to a therapeutic agent (e.g.,
drug, toxin, enzyme or second antibody) or in a
recombinant form (e.g., chimeric or bispecific BR96).
The compositions may additionally include other

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antibodies or conjugates for treating carcinomas (e.g.,
an antibody cocktail).

The antibody compositions of the invention can
be administered using conventional modes of
administration including, but not limited to,
intravenous, intraperitoneal, oral, intralymphatic or
administration directly into the tumor. Intravenous
administration is preferred.

The antibody compositions of the invention may
be in a variety of dosage forms which include, but are
not limited to, liquid solutions or suspensions, tablets,
pills, powders, suppositories, polymeric microcapsules or
microvesicles, liposomes, and injectable or infusible
solutions. The preferred form depends upon the mode of
administration and the therapeutic application.

The antibody compositions also preferably
include conventional pharmaceutically acceptable carriers
and adjuvants known in the art such as human serum
albumin, ion exchangers, alumina, lecithin, buffer
substances such as phosphates, glycine, sorbic acid,
potassium sorbate, and salts or electrolytes such as
protamine sulfate.

The most effective mode of administration and
dosage regimen for the compositions of this invention
depends upon the severity and course of the disease, the
patient's health and response to treatment and the
judgment of the treating physician. Accordingly, the
dosages of the compositions should be titrated to the
individual patient. Nevertheless, an effective dose of
the antibody compositions of this invention may be in the
range of from about 1 to about 2000 mg/m^2.

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In order that the invention described herein may be more fully understood, the following examples are set forth. It should be understood that these examples are for illustrative purposes only and are not to be construed as limiting the scope of this invention in any manner.

EXAMPLE 1

Preparation Of The BR96 Monoclonal Antibody

The BR96 monoclonal antibody of the invention was produced using hybridoma fusion techniques as described previously by M. Yeh et al., Proc. Natl. Acad. Sci. USA, (1979), supra and Yeh et al., Int. J. Cancer (1982), supra. Briefly, a three month-old BALB/c mouse was immunized using as the immunogen explanted cultured cells from a human breast adenocarcinoma, designated 3396 or H3396 (from adenocarcinoma of the breast from a patient which had been established in culture at Oncogen, Seattle, Washington). The mouse received injections on five occasions: on the first four occasions, the mouse received one intraperitoneal injection and 1 subcutaneous injection split between 4 sites on the mouse. On the fifth occasion, the mouse was given only one intraperitoneal injection. The total number of cells injected on each occasion was approximately 10^7 cells. Three days after the last immunization, the spleen was removed and spleen cells were suspended in RPMI culture medium. The spleen cells were then fused with P3-x63-Ag8.653 mouse myeloma cells in the presence of polyethylene glycol (PEG) and the cell suspension grown in microtiter wells in selective HAT medium as described by Yeh et al., supra [see, also, Kohler and Milstein, Nature, 256:495-97 (1975) and Eur. J. Immunol., 6:511-19 (1976)]. The mixture was seeded to form low density cultures originating from single fused cells or clones.

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The supernatants from these hybridoma cultures were then screened for direct binding activity on the breast cancer cell line, 3396, and a fibroblast cell line obtained from a skin biopsy using an ELISA assay similar to that described by Douillard et al., "Enzyme-Linked Immunosorbent Assay For Screening Monoclonal Antibody Production Using Enzyme-Labeled Second Antibody", Meth. Enzymol., 92:168-74 (1983).

According to this assay, the antigen (with which the antibody being screened for is reactive) is immobilized on microtiter plates and then incubated with hybridoma supernatants. If a supernatant contains the desired antibody, the antibody will bind to the immobilized antigen and is detected by addition of an anti-immunoglobulin antibody-enzyme conjugate and a substrate for the enzyme which leads to a measurable change in optical density. In the present studies, breast cancer cells or control fibroblast cells were dispensed into a 96-well tissue culture plate (Costar Cambridge, MA) and incubated overnight in a humid 37°C incubator (5% CO_2). The cells were then fixed with 100 μl of freshly prepared 1.0% glutaraldehyde to a final well concentration of 0.5% and incubated for 15 min at room temperature, followed by washing three times with 1 X phosphate buffered saline (PBS). The cells were next blocked for 30 min with 5% bovine serum albumin (BSA) in PBS and washed again three times with PBS. The supernatants from the hybridoma cultures were then added at 100 μl/well, the wells incubated for 1 h at room temperature, and the cells washed three times with PBS. Next, goat anti-mouse horseradish peroxidase (Zymed, CA) diluted in 0.1% BSA and PBS was added to a concentration of 100 μl/well. The reaction mixture was incubated for either 1 h at room temperature or 30 min at 37°C and the cells were then washed three times with PBS. o-Phenylenediamine (OPD) was then added at 100 μl/well and

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the plates incubated in the dark at room temperature for
5-45 min. Antibody binding to the cells was detected by
a color change in the wells that occurred within 10-20
min. The reaction was stopped by adding 100 μl/well H_2SO_4
and the absorbance read in a Dynatech (Alexandria, VA)
Microelisa autoreader at 490 nm.

It should be noted that this assay can be
performed using intact cells or purified soluble antigen
or cellular extracts as the immobilized antigen. When
soluble antigen or cell extracts were used as antigen,
the antigen was initially plated at 50 μl/well in PBS and
the plates were incubated overnight at room temperature
before beginning the assay. When using intact cells as
antigen, they may be used fresh or after fixation. In
either case, the cells were initially plated at 10^4 cells
in 100 μl/well in culture medium and incubated overnight
in a 37°C incubator (5% CO_2).

Hybridomas which produced antibodies binding to
the breast cancer cell line and not to the human
fibroblast cells were thus selected, and tested in a FACS
cell sorter on peripheral blood leukocytes (PBLs), as
described in Example 2, _infra_. Hybridomas that were
negative on PBLs were cloned, expanded _in vitro,_ and
further tested for antibody specificity. Those
hybridomas producing antibody reactive with human breast
cancer were recloned, expanded, and injected into
pristane-primed 3-month old BALB/c mice, where they grew
as ascites tumors.

Following this procedure, hybridoma cell line
BR96 was obtained, cloned and injected into mice to
develop as an ascites tumor. As disclosed above, the
BR96 hybridoma has been deposited with the ATCC.
Monoclonal BR96 antibody was purified from ascites by
affinity chromatography on immobilized recombinant

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protein A (Repligen, Cambridge, MA). Clarified ascites
was diluted with an equal volume of binding buffer (1 M
potassium phosphate, pH 8) and applied to a protein A
column previously equilibrated with binding buffer. The
column was extensively washed with binding buffer and
then the antibody was eluted with 50 mM phosphoric acid,
pH 3. The purified antibody fraction was neutralized
with 1 M Tris, pH 9 and then dialyzed against phosphate
buffered saline. Purified BR96 was finally sterile
filtered and stored refrigerated or frozen.

EXAMPLE 2

Characterization Of The BR96 Monoclonal Antibody

Isotype Determination

To determine the class of immunoglobulin
produced by the BR96 hybridoma, the following techniques
were utilized:

(a)   Ouchterlony Immunodiffusion

An aliquot of supernatant of the hybridoma
cells was placed into the center well of the a 25% agar
plate. Monospecific rabbit anti-mouse Ig isotype
antibodies (Southern Biotechnology, Birmingham, AL) were
placed in the outer wells and the plate was incubated for
24-28 h at room temperature. Precipitation lines were
then read.

(b)   ELISA Isotyping

Dynatech Immulon 96-well plates were coated
with goat anti-mouse Ig antibodies at 1 μg/ml
concentration, 50 μl/well in PBS and left covered
overnight at 4°C. The plates were washed with PBS/Tween
20, 0.05% and blocked with medium at 100 μl/well for 1 h

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at room temperature.   After washing the plates,
supernatants from the BR96 hybridoma were added and
incubated at room temperature for 1 h.   After washing
with PBS containing 2% bovine serum albumin (BSA), plates
were incubated at 37°C for 30 min with monospecific
rabbit anti-mouse Ig isotype antibodies coupled to
peroxidase (Zymed, South San Francisco, CA).   After
further washing, the plates were incubated with 1 mg/ml
OPD and 0.03% H_2O_2 in 0.1 M citrate buffer, pH 4.5.
Optical density at 630 nm was determined on a Dynatec
ELISA plate reader.

Based on these procedures, it was determined
that the BR96 monoclonal antibody is of the IgG3 isotype.

Characteristics Of The BR96 Monoclonal Antibody

The BR96 antibody shows a high degree of
reactivity with a wide range of carcinomas and displays
only limited reactivity with normal cells. This was
shown by experiments involving immunohistological studies
on frozen tissue sections as well as binding studies
using intact cultured cells.

Immunohistology

The peroxidase-antiperoxidase (PAP) technique
of L.A. Sternberger as described in Immunochemistry, pp.
104-69 (John Wiley & Sons, New York, 1979) and as
modified by H.J. Garrigues et al., "Detection Of A Human
Melanoma-Associated Antigen, p97, In Histological
Sections Of Primary Human Melanomas", Int. J. Cancer,
29:511-15 (1982), was used for the immunohistological
studies. The target tissues for these tests were obtained
at surgery and frozen within 4 h of removal using
isopentane precooled in liquid nitrogen. Tissues were
then stored in liquid nitrogen or at -70°C until used.

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Frozen sections were prepared, air dried, treated with acetone and dried again [see Garrigues et al., supra]. Sections to be used for histologic evaluation were stained with hematoxylin. To decrease non-specific backgrounds sections were preincubated with normal human serum diluted 1/5 in PBS [see Garrigues et al., supra]. Mouse antibodies, rabbit anti-mouse IgG, and mouse PAP were diluted in a solution of 10% normal human serum and 3% rabbit serum. Rabbit anti-mouse IgG (Sternberger-Meyer Immunochemicals, Inc., Jarettsville, MD), was used at a dilution of 1/50. Mouse PAP complexes (Sternberger-Meyer Immunochemicals, Inc.) containing 2 mg/ml of specifically purified PAP was used at a dilution of 1/80.

The staining procedure consisted of treating serial sections with either specific antibody, i.e., BR96, or a control antibody for 2.5 h, incubating the sections for 30 min at room temperature with rabbit anti-mouse IgG diluted 1/50 and then exposing the sections to mouse PAP complexes diluted 1/80 for 30 min at room temperature. After each treatment with antibody, the slides were washed twice in PBS.

The immunohistochemical reaction was developed by adding freshly prepared 0.5% 3,3'-diaminobenzidine tetrahydrochloride (Sigma Chemical Co., St. Louis, MO) and 0.01% H_2O_2 in 0.05 M Tris buffer, pH 7.6, for 8 min [see Hellstrom et al., J. Immunol., 127:157-60 (1981)]. Further exposure to a 1% OsO_4 solution in distilled water for 20 min intensified the stain. The sections were rinsed with water, dehydrated in alcohol, cleared in xylene, and mounted on slides. Parallel sections were stained with hematoxylin.

The slides were each evaluated under code and coded samples were checked by an independent investigator. Typical slides were photographed by using

differential interference contrast optics
(Zeiss-Nomarski). The degree of antibody staining was
evaluated as 0 (no reactivity), + (a few weakly positive
cells), ++ (at least one third of the cells positive),
+++ (most cells positive), ++++ (approximately all cells
strongly positive). Because differences between + and 0
staining were less clear cut than between + and ++
staining, a staining graded as ++ or greater was
considered "positive". Both neoplastic and stroma cells
were observed in tumor samples. The staining recorded is
that of the tumor cells because the stroma cells were not
stained at all or were stained much more weakly than the
tumor cells.

Table 1 below demonstrates the
immunohistological staining of various tumor and normal
tissue specimens using the BR96 monoclonal antibody. As
the table clearly demonstrates, the BR96 antibody reacts
with a wide range of human carcinoma specimens, does not
react with sarcoma and displays only infrequent
reactivity with melanoma. Furthermore, it shows only
limited reactivity with any of the large number of normal
human tissues tested. The only reactivity detected with
normal cells detected was binding to a small
subpopulation of cells in the tonsils and in the testis,
and to acinar cells in the pancreas, and to epithelial
cells of the stomach and esophagus.

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TABLE 1

Immunoperoxidase Staining of Human Tumors and Normal Tissue Specimens with BR96 Monoclonal Antibody

TISSUE TYPE	NUMBER POSITIVE/NUMBER TESTED
Tumors	
Lung carcinoma (non-small cell)	14/17
Breast carcinoma	17/19
Colon carcinoma	15/18
Ovary carcinoma	4/ 4
Endometrial carcinoma	2/ 2
Melanoma	2/ 5
Sarcoma	0/ 5
Stomach carcinoma	2/ 2
Pancreatic carcinoma	2/ 2
Esophagus carcinoma	2/ 2
Cervical carcinoma	2/ 2
Normal Tissues	
Lung	0/ 7
Spleen	0/ 5
Breast	0/ 2
Colon	0/ 7
Kidney	0/ 7
Liver	0/ 5
Brain	0/ 2
Heart	0/ 3
Skin	0/ 2
Thyroid	0/ 2
Adrenal	0/ 1
Ovary	0/ 2
Lymph nodes	0/ 2
Lymphocyte pellet	0/ 4

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Table 1 (continued)
Immunoperoxidase Staining of Tumors and Normal
Tissue Specimens with BR96 Monoclonal Antibody

TISSUE TYPE	NUMBER POSITIVE/NUMBER TESTED
Pancreas	2/ 2 (only acinar cells were positive)
Uterus	0/ 7
Retina	0/ 1
Testis	2/ 2 (only small sub-population of cells were positive)
Tonsil	2/ 2 (only small sub population of cells were positive)
Stomach	2/ 2 (epithelial cells positive)
Esophagus	2/ 2 (epithelial cells positive)

The binding of the BR96 antibody to various cultured cell lines was also evaluated. Antibody binding to the cell surface of intact cultured cells was identified either by a direct binding assay with ^{125}I-labeled antibody as described in Brown et al., "Quantitative Analysis Of Melanoma-Associated Antigen p97 In Normal And Neoplastic Tissues", Proc. Natl. Acad. Sci. USA, 78:539-43 (1981), or by direct immunofluorescence using a Coulter Epics C fluorescence activated cell sorter (FACS) II [Hellstrom et al., Cancer Res. 46:3917-3923 (1986)].

For binding analyses using a FACS cell sorter, 2×10^5 to 1×10^6 cultured cells were aliquoted in 15% fetal bovine serum (FBS) in IMDM media (Gibco, NY) to a

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total volume of 500 μl /tube.   The cells were centrifuged
for 1.5 min on a Serofuge and the supernatant removed.
100 μl of the BR96 monoclonal antibody at 10 μl/ml was
added to each tube, the contents of which was then mixed
and incubated on ice for 30 min.   The reaction mixture
was washed three times with 500 μl of 15% FBS/IMDM by
centrifugation for 1.5 min on the Serofuge (tubes were
blotted after the third wash).   Then, 50 μl of optimized
FITC-conjugated goat anti-mouse IgG antibody (Tago,
Burlingame, CA) diluted 1:25 in 15% FBS/IMDM was added to
each tube and the reaction mixture was mixed and
incubated for 30 min.   The wash step was then repeated
and after blotting of the tubes, each pellet was
resuspended in 200-500 μl of PBS.   Each sample was run on
a Coulter Epics C FACS and the mean fluorescence
intensity (MFI) was determined.   From the MFI, the linear
fluorescent equivalent (LFE) was determined.   The LFE of
each test sample divided by the LFE of a negative control
gave a ratio between the brightness of cells stained by
specific versus control antibody.   The binding data is
shown in Table 2 below.

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TABLE 2

FACS Analysis of the Binding of BR96 to
Various Types of Suspended Cells

Cell line		Ratio (10 μg/ml)
Breast carcinoma	3396	54
Breast carcinoma	MCF-7	38
Breast carcinoma	3630	22
Breast carcinoma	3680	22
Lung carcinoma	2987	15
Lung carcinoma	2707	30
Lung carcinoma	2964	2
Lung carcinoma	3655-3	18
Colon carcinoma	RCA	34
Colon carcinoma	3619	22
Colon carcinoma	3347	5
Colon carcinoma	HCT116	1
Colon carcinoma	CB5	27
Colon carcinoma	C	30
Colon carcinoma	3600	16
Ovary carcinoma	3633-3	11
Melanoma	2669	1
Melanoma	3606	1
Melanoma	3620	1
T cell lymphoma line	CEM	1
T cell lymphoma line	MOLT-4	1
B cell lymphoma line	P3HR1	1
Peripheral blood leukocytes		1

As Table 2 demonstrates, the BR96 monoclonal antibody reacted with breast, lung and colon carcinoma cell lines but did not react with melanoma lines or with

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T or B lymphoma lines nor with normal peripheral blood leukocytes. Scatchard analysis using radiolabeled antibody indicated that the approximate association constant (K_a) of BR96 was calculated to be 3.6 X 10^6 antigen sites/cell for the 3396 line which binds BR96.

These data demonstrate that monoclonal antibody BR96 recognize cell surface antigens abundantly expressed (up to 10^6 molecules/cell) on the majority of human carcinomas.

EXAMPLE 3

Internalization Of The BR96 Monoclonal Antibody Within Carcinoma Cells

Studies were conducted to measure internalization of the BR96 monoclonal antibody within antigen-positive carcinoma cells. According to one procedure, BR96 was conjugated to the ricin A chain toxin to form an immunotoxin, BR96-RA, whose internalization by carcinoma cells was then determined. Uptake of the conjugate by the carcinoma cells was assessed by determining to what extent the tumor cells were killed by ricin A chain.

Conjugation of the antibody to the toxin was carried out as follows: Deglycosylated ricin-A chain (Inland Labs, Austin, TX) [see, also, Blakey et al., Cancer Res., 47:947-952 (1987)] was treated with dithiothreitol (5 mM) prior to gel filtration on G-25 Sephadex using PBS, pH 7.2 as eluant. This was added in a 2:1 molar ratio to the antibody in PBS, the antibody having been previously modified with N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP) (Pierce, Rockford, IL) according to the procedure of Lambert et al., J. Biol. Chem., 260:12035-12041 (1985). Reaction was allowed to

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proceed for 12-24 h at room temperature, and the solution
was then diluted with 1 volume of H$_2$O. Removal of
unconjugated antibody was achieved using Blue Sepharose
CL-6B (Pharmacia, Uppsala, Sweden) [see Knowles et al.,
Anal. Biochem., 160:440-443 (1987)].

The conjugate and excess ricin-A chain were
eluted with high salt (10 x PBS) and subjected to further
purification on Sephacryl-300 (Pharmacia) using PBS as
eluant. The resulting conjugate was free of unbound
monoclonal antibody or ricin A-chain and consisted mostly
of 1:1 adducts.

The internalization of BR96-RA by various
carcinoma cell lines was then measured using a thymidine
uptake inhibition assay. According to this assay, the
inhibition of ^{3}H-thymidine incorporation into the DNA of
the carcinoma cells (i.e., the inhibition of cell
proliferation) is a measure of the cytotoxic effect of
BR96-RA on the cells and thus a measure of the
internalization of the immunotoxin within the cell.

For the assay, carcinoma cells were plated into
a 96-well microtiter plate at 1 x 10^4 cells/well in 100 μl
of IMDM medium with 15% fetal calf serum (FCS). The
plates were incubated for 12-18 h at 37°C to let the
cells adhere. Then the media was removed. Plates were
kept on ice. The BR96-RA immunotoxin (100 μl) was then
added in log 10 serial dilutions, starting at 10 μg/ml
final concentration down to 0.01 μg/ml. The reaction
mixture was incubated for 4 h on ice. The plates were
washed and 200 μl/ml media was added and further
incubated at 37°C for 18 h. At this point, 50 μl of
^{3}H-thymidine was added at 1 μCi/well and the plates
incubated for 6 h at 37°C in a 5% CO$_2$ incubator. The
assay plates were then frozen at -70°C for at least 1 h
and thawed in a gel dryer for 15 min. The cells were
harvested onto glass fiber filters (Filter Strips, No.

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240-1, Cambridge Technology) in plastic scintillation
vials using a PHD cell harvester. 3 ml of scintillation
counting liquid was added to the vials and the vials were
counted on a Beckman LS3891 beta scintillation counter at
1 minute per sample.

Graphs of the percent inhibition of thymidine
incorporation vs. immunotoxin concentration for each cell
line tested were plotted and are shown in Figures 1-5.
In each assay, a control was run. The results of the
assay are expressed as a percentage of the ^3H thymidine
incorporated by untreated control cells.

Figure 1 depicts the percent inhibition of
thymidine incorporation by cells from the 3396 breast
carcinoma cell line caused by internalization of BR96-RA.
Similar results were obtained with the 2707 lung
carcinoma cell line (Figure 2) and C colon carcinoma cell
line (see Figure 4). The BR96-RA was not internalized by
HCT 116 cell line, a human colon carcinoma cell line that
does not bind BR96 (see Figure 3). Figure 5 shows no
internalization of BR96-RA on 3347, a colon carcinoma
cell line to which BR96 does not bind; BR6-RA, on the
other hand, which binds to the 3347 cells, does
internalize. This study, therefore, demonstrated not
only internalization of the BR96 antibody but the
selectivity of the internalization of the BR96 antibody
for antigen positive carcinoma cells.

EXAMPLE 4

Cytotoxicity of Unmodified BR96 Monoclonal Antibody

Three types of experiments were performed to
follow up on the unexpected observation that monoclonal
antibody BR96 appeared to be cytotoxic by itself (i.e.,
in unmodified state) when tested in a FACS assay. So as
to avoid an effect of complement in serum, all sera used

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were heat inactivated (56°C for 30 min); in addition, some
of the experiments with FACS analysis (as described
below) were performed on cells which were grown in serum-
free medium and tested in the absence of serum.

First, living suspended cells from a variety of
antigen positive carcinoma lines (3396, 2987, 3619) were
treated with monoclonal antibody BR96. Cells (5 x 10^5)
were incubated on ice for 30 min with 100 μl of BR96 or
control monoclonal antibody at a concentration of 60, 30,
15, 7.5 and 3.8 μg/ml in culture medium (IMDM, 15% FBS).
After washing the cells twice with culture medium, the
cells were suspended in 500 μl medium and stained by
adding the dye propidium iodide which stains dead cells
[Krishan, Cell Biol. 66:188 (1975); and Yeh, J. Immunol.
Methods, 43:269 (1981)]. Out of a 1 mg/ml stock solution
(in 70% alcohol) 5 μl dye was added to cell samples,
incubated on ice for 15 min, washed once and finally
suspended in 500 μl medium. The cells were evaluated on
a Coulter Epics C FACS, with dead cells being identified
by their red fluorescence. The analysis was done on a
two-parameter display with log forward lightscatter in
the horizontal and log red fluorescence in the vertical
display. Computations of cell size versus cell viability
were obtained by applying the Coulter Epics C Quadstat
program. Tumor cells which could bind BR96 as well as
tumor cells not binding BR96

were studied in parallel. The results are shown in
Figure 6. Figure 6 demonstrates that incubation of cells
from any of three antigen-positive carcinomas with BR96
rapidly killed them. Untreated or antigen-negative cells
were not killed.

Second, tumor cells (3396, 3630, 2987, 3619 and
HCT 116) were exposed to BR96 (or the control monoclonal
antibody) for 18 h at 37°C in a 96-well microtiter plate

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at 3 X 10³ cells/well in 150 μl of IMDM medium containing
FBS for 66 h after which 50 μl of ³H-thymidine was added
at 1 μCi/well and the plate was incubated for another 6 h
at 37°C. Subsequently, it was frozen at -70°C for at
least 1 h and thawed in a gel dryer for 15 min, and the
cells harvested onto glass fiber filters. The tritiated
thymidine assay was then performed as described in the
preceding example, except that the cells and antibodies
were incubated at 37°C. Figure 7 illustrates the
results. BR96 caused an inhibition of [³H]thymidine
incorporation into antigen-positive cell lines, and this
effect was dose dependent. The antigen-negative cell
line HCT116 was not affected by an concentration of BR96.

Third, using a modification of a procedure
described by Linsley et al. [Linsley, et al.,
"Identification and characterization of cellular
receptors for growth regulator, Oncostatin M", J. Biol.
Chem. 264:4282-4289 (1989)] a growth inhibition assay was
performed. Cells from four different cell lines (HCT116,
2987, 3396 and 3630) were seeded (3 x 10³) in a volume of
0.1 ml of IMDM with 15% fetal bovine serum (FBS) in 96-
well microtiter plates and allowed to attach for 3 h at
37°C. Various concentrations of whole BR96 monoclonal
were then added in a volume of 0.1 ml, after which
incubation at 37°C was continued for 72 h. Subsequently,
the culture medium was removed and the cells were stained
by crystal violet (0.1% in 20% methanol) for 30 min. and
washed three times with PBS. The bound dye was eluted by
the addition of 0.1 ml of a solution of 0.1 M sodium
citrate, Ph 4.2, in 50% ethanol. Samples were assayed in
triplicate on an ELISA reader measuring the absorbance in
the presence of BR96 with the absorbance in untreated
samples. The results of this procedure are expressed as
percentage inhibition of cell growth. Figure 8
illustrates the results. The results of this assay were

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in agreement with those presented above for the thymidine incorporation assay (Figure 7).

EXAMPLE 5

ADCC Activity of BR96 Antibody

Determination of ADCC activity of BR96 monoclonal antibody was performed as described by Hellstrom et al., Proc. Natl. Acad. Sci. (USA) 82:1499-1502 (1985). Briefly, a short-term ^{51}Cr-release test that measures the release of ^{51}Cr as described by Cerrotini et al., Adv. Immunol. 18:67-132 (1974) was used as evidence of tumor-cell lysis (cytotoxicity). Peripheral blood lymphocytes from healthy human subjects were separated on Ficoll-Hypaque [Hellstrom et al., Int. J. Cancer 27:281-285 (1981)] to provide effector cells equal to 5% natural killer cell reactivity against SK-MEL-28 cells; (10^6) cells were labeled by incubation with 100 μCi (1 Ci = 37 Gbq) of ^{51}Cr for 2 h at 37°C, after which they were washed three times and resuspended in medium. The labeled cells were seeded (2 X 10^4 cells per well in 20 μl) into Microtiter V-bottom plates (Dynatech Laboratories, Alexandria, VA). Purified antibody BR96 (10 μg/ml, 1 μg/ml, and 0.1 μg/ml) was then added, followed by 2 X 10^5 lymphocytes per well in 100 μl. The mixtures were incubated for 2 to 4 h after which the plates were centrifuged at 400 X g. The supernatants were removed and the radioactivity in 100 μl samples was measured with a gamma-counter. There were two replicates per group; the variation between replicates was less than 10%. Several "criss-cross" experiments were done, in which lung (or colon) carcinoma and melanoma targets were tested in parallel with monoclonal antibody BR96 and with the antimelanoma monoclonal antibody MG-22 [Hellstrom et al., Proc. Natl. Acad. Sci. USA, 82:1499-1502 (1985)] which do not bind to most carcinoma cells. Controls

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included the incubation of target cells alone or with
either lymphocytes or monoclonal antibody separately.

Spontaneous release was defined as the counts
per minute (cpm) released into the medium from target
cells exposed to neither antibodies nor lymphocytes, and
total release, as the number of counts released from
target cells that were osmotically lysed at the end of
the assay. Percent cytotoxicity was calculated as:

$$\frac{\text{experimental group release} - \text{spontaneous release}}{\text{total release} - \text{spontaneous release}} \times 100$$

Effector cells were characterized by assessing
their sensitivity to incubation with anti-serum to the
Leu-11b surface marker and guinea pig complement, using
procedures described by Hellstrom et al., in Monoclonal
Antibodies and Cancer Therapy, UCLA Symposia on Molecular
and Cellular Biology, New Series, eds. Reisfeld & Sell,
Liss, New York, Vol 27, pp. 149-164 (1985), incorporated
herein by reference. This was done to measure the
expression of the Leu-11b marker, which characterizes
natural killer (NK) cells and is expressed by lymphocytes
mediating ADCC against human melanoma cells in the
presence of monoclonal antibody BR96. The cytotoxicity
by effector cells alone ("natural killer effect") was
subtracted from the data provided in Figure 9.

The results shown in Figure 9 for an antibody
concentration of 10 μg/ml indicate that BR96 mediates
ADCC activity if present in sufficient concentrations and
if the target cells express sufficient concentrations of
the epitope. The ADCC activity can be seen at antibody
concentrations lower than those at which the antibody is
cytotoxic by itself (usually around 20 μg/ml). When
antibody BR96 was used alone as a control it produced 0%
killing at the concentrations tested and using the ^{51}Cr

assay. ADCC activity was only found with BR96 antibody-
binding cell lines. Thus, cells from five different
carcinoma lines, which all bound BR96, were killed via
ADCC at monoclonal antibody concentrations down to 0.1
µg/ml, while cells from a sixth line, 2964, which did not
bind BR96, were not killed. The requirement for antibody
binding to obtain ADCC was further demonstrated by the
fact that both of the two carcinomas which could bind a
different antibody, L6 (lines 3619 and 2987), were killed
by L6 via ADCC, while the others were not. Under the
conditions of the assay, BR96 alone caused the release of
only 1% of the label, even when tested at a concentration
of 10 µg/ml.

                            EXAMPLE 6

Ability of BR96 to Mediate Complement-Mediated
Cytotoxicity (CDC)

        Tests to evaluate the ability of monoclonal
antibody BR96 to kill tumor cells in the presence of
human serum as a source of complement (complement-
mediated cytotoxicity) (CDC) were performed similarly to
those for the ADCC tests described in Example 5, supra,
except that 100 µl of human serum from normal human
subjects as the source of complement diluted 1:3 to 1:6
was added per microtest well in place of a suspension of
effector cells.

        As shown in Figure 10, CDC against cells binding
BR96 was seen at an antibody concentration of 0.1-5.0
µg/ml, while there was no CDC against the BR96 antigen-
negative lines HCT116 and 3347. The 3347 cells could,
however, be killed when using the L6 monoclonal antibody,
which binds to these cells. Controls were always
included in which BR96 was tested in the absence of
complement. No killing by BR96 alone was detected by the

[51]Cr release assay. These data show that BR96 gave a cytotoxic effect in the presence of human serum at concentrations where it is not cytotoxic by itself. (Control antibody gave no CDC).

EXAMPLE 7
Determination of Reactivity of BR96 to Glycolipids and Glycoproteins

BR96 antibody was tested for reactivity to a variety of immobilized glycolipid antigens having known carbohydrate structures and synthetic glycoproteins (so called "neoglycoproteins") using an ELISA assay in which purified glycolipids and glycoproteins and antibody were used in excess (Dr. John Magnani, Biocarb, Gaithersburg, MD; Lloyd et al., Immunogenetics 17:537-541 (1983)). Glycolipids were dried from methanol in microliter wells at 100 ng/well. Synthetic glycoproteins were coated on the surface of the wells by incubation of glycoprotein diluted to 200 ng in phosphate buffered saline (PBS), at pH 7.4/well. Purified BR96 was assayed at a concentration of 10 μg/ml in 0.01 M Tris-HCl, pH 7.4, containing 1% BSA containing 1% bovine serum albumin and antibodies from ascites were assayed at a dilution of 1:100 in the same buffer. At these high concentrations most binding interactions are readily detected. Absorbance values were calculated as the average of duplicate wells. The results of this analysis are summarized in Figures 11 and 12 showing that BR96 reacted with Le[Y] antigen.

These findings indicate that BR96 can bind to a variant form of the Lewis Y (Fuc α1-2Galβ1-4(Fucα2-3)GlcNAc. antigen and that fucose α1-3 attached to GlcNAc forms a portion of the Le[Y]-related epitope recognized by BR96. The high tumor specificity of BR96 and ability to internalize (not previously described for monoclonal

antibodies reactive with Le^y antigens) suggests that the
antibody recognizes a complex epitope, a portion of which
includes the Le^y antigen.

EXAMPLE 8

Preparation and Characterization of BR96 F(ab')$_2$ Fragments

Murine BR96 (IgG$_3$) was purified by Protein A
affinity chromatography from murine ascitic fluid.
Briefly, delipidated ascites was passed over a column
containing a matrix of immobilized Protein A (RepliGen
Corp., Cambridge, MA) previously equilibrated with 1 M
potassium phosphate, pH 8.0. Following the passage of
ascites, the column was washed with equilibration buffer
until no further protein was spectrophotometrically
detected. The bound BR96 was then eluted from the column
using 0.1 M citrate buffer, pH 3.0. Immediately after
elution, the eluate was neutralized with
1.0 M Tris buffer, pH 9.0, until the pH was approximately
7.0. The monoclonal antibody was then dialyzed into PBS
and concentrated prior to storage or use.

F(ab')$_2$ fragments were then generated by
digesting purified BR96 monoclonal antibody with pepsin
according to Lamoy, "Preparation of F(ab')$_2$ Fragments from
Mouse IgG of Various Subclasses", Meth. Enzymol. 121:652-
663 (1986). Residual whole antibody and Fc fragments were
adsorbed from the reaction mixture by passage over a
protein A affinity column. The resulting F(ab')$_2$ fragment
preparations were dialyzed extensively against PBS and
sterile filtered.

The BR96 F(ab')$_2$ fragments preparations were
characterized by gel permeation HPLC, SDS-PAGE and by
ELISA on the human breast tumor line 3396 (Oncogen,
Seattle, WA). Gel permeation HPLC was used to assess the

molecular sizes of the proteins comprising the F(ab')$_2$
preparation. Reproducible chromatograms from different
preparations indicated that 75-80% of the protein was
F(ab')$_2$. No protein was detected at the positions
representing higher molecular weight material, such as
whole BR96 or protein aggregates. The remaining 20-25%
of the protein eluted at positions corresponding to
inactivated pepsin and to other smaller non-protein A-
binding digestion products.

Nonreducing and reducing SDS-PAGE was used to
examine the denatured molecular sizes and structural
arrangement of the proteins in the F(ab')$_2$ preparations.
A single major band at the position of F(ab')$_2$
(approximately 100 kdal) was typically observed, with no
visible contaminating whole monoclonal antibody band (160
kdal). Lower molecular weight bands (i.e. less than 100
kdal) representing inactivated pepsin and small digestion
products were minimal. Under reducing conditions the
expected results were obtained with the only major bands
occurring as a doublet at approximately 25 kdal
representing the light chain and the remaining fragmented
portion of the heavy chain. No whole heavy chain band
was observed.

Functional (binding) activity of the BR96
F(ab')$_2$ fragments was compared to that of whole BR96 in an
ELISA with 3396 cells supplying the antigen. Binding of
BR96 whole antibody or F(ab')$_2$ fragments to the cells was
detected with an HRP-conjugated goat anti-murine K light
chain reagent as shown in Figure 13. On a duplicate
plate, binding of whole BR96 was distinguished from
binding of F(ab')$_2$ fragments by using HRP-conjugated
protein A which binds to the whole antibody but not the
F(ab')$_2$ fragments (Figure 14).

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These results indicate that BR96 F(ab')$_2$ (lot R0201-1663-03, lot 2) contained a trace amount of whole BR96 antibody. The level of contaminating whole antibody can be estimated to be approximately 8 trifold dilutions away from the amount of F(ab')$_2$ present, or about 0.01%. The other F(ab')$_2$ preparation (lot R9011-1481-48, lot 1) showed no detectable level of contaminating whole BR96, indicating that any effect of BR96 can be explained by binding of the Fab region and not the Fc region.

In summary, the BR96 F(ab')$_2$ preparations appear to be completely free of contaminating whole BR96 IgG by HPLC and by SDS-PAGE. In only one instance, when a very sensitive ELISA method was used were detectable levels of contaminating whole BR96 antibody found and this represented only approximately 0.01% by weight compared to the amount of F(ab')$_2$ fragments present.

EXAMPLE 9

Preparation and Characterization of Chimeric BR96 Antibody (ChiBR96)

The murine/human chimeric BR96 antibody of the invention ("ChiBR96") was produced using a two-step homologous recombination protocol as described by Fell et al., in Proc. Natl. Acad. Sci. USA 86:8507-8511 (1989) and in co-pending patent application by Fell and Folger-Bruce, U.S. Serial No. 243,873, filed September 14, 1988, and Serial No. 468,035, filed January 22, 1990, and assigned to the same assignee as the present application; the disclosures of all of these documents are incorporated in their entirety by reference herein.

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Human Heavy Chain DNA Transfection

The murine hybridoma cell line BR96, ATCC No. HB10036, obtained as described above was transfected (8 X 10^6 cells) with hgamma1/HC-D (deposited at Agricultural Research Service Culture Collection (NRRL), Peoria, Illinois, NRRL No. B 18599) (Figure 15) by electroporation (Gene Pulser; Biorad Laboratories, Richmond, CA) at 250 V, 960 μFd capacitance setting, in isotonic phosphate buffered saline (PBS) and 30 μg/ml of the purified 6.2 kb Xba1 restricted fragment of the vector hgamma1HC-D. After 48 hr cells were seeded in 96-well plates at 10^4 cells/well. Selection for Neo^R was carried out in IMDM medium (GIBCO, Grand Island, NY) containing 10% (vol/vol) fetal bovine serum (FBS) and the antibiotic aminoglycoside G418 (GIBCO) at 2.0 mg/ml.

Detection of Secreted Human IgG (Hu gamma1) Antibody by ELISA

Culture supernatants were screened using a sandwich ELISA assay 2 weeks after transfection. Goat anti-human IgG, Fc specific (CALTAG, San Francisco, CA) was used as the capture antibody and goat αhuman IgG, Fc specific conjugated to horseradish peroxidase HRPO, (CALTAG) was the antibody used to detect bound human IgG. Cells from the HuIgG positive wells were subcloned by dilution and dilution clones were screened by ELISA to detect human IgGgamma1 by the previously described method. The clones containing human IgGgamma1 were also screened by ELISA to detect murine IgG3 heavy chain. Goat anti-mouse IgG3 (Southern Biotechnology Assoc., Inc., Birmingham, AL) was used as the capture antibody and goat anti-mouse conjugated to HRPO (Southern Biotechnology Assoc., Inc.) was the antibody used to detect the mouse IgG3.

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One of the human IgGgamma1 positive murine IgG3 negative (Hugamma1+, MuG3-) clones was chosen and designated ChiHBR96. This heavy chain chimeric hybridoma cell line, ChiHBR96 was characterized for antigen specificity on MCF-7 cells and for expression levels by a quantitative ELISA for human IgG expression on MCF7 cells. The cell line ChiHBR96 expressed approximately 20 $\mu g/ml$ of antigen-specific human IgG antibody.

Light Chain DNA Transfection

The ChiHBR96 hybridoma (8 X 10^6 cells) was transfected by electroporation as described above but using 30 $\mu g/ml$ of the human light chain recombination vector pSV₂gpt/C_K (NRRL No. B 18507) containing the human light chain K immunoglobulin sequence shown in Figure 16, linearized with HindIII. After 48 hr cells were seeded in 96-well plates at 10^4 cells/well. Selection for gpt was carried out in IMDM medium containing 10% (vol/vol) FBS, 15 $\mu g/ml$ hypoxanthine, 250 $\mu g/ml$ xanthine and 2.25 $\mu g/ml$ mycophenolic acid (MA).

Detection of Secreted Human Kappa (Hu K) Antibody by ELISA

Culture supernatants were screened using a sandwich ELISA assay as described above, 2 weeks after transfection. Goat α-human K (CALTAG) was the capture antibody and goat anti-human K HRPO (CALTAG) was the antibody used to detect bound human K. Wells containing human K antibody were subcloned by dilution and the clones were screened by ELISA to detect human K or murine K chain. Goat anti-mouse K (Fisher Scientific, Pittsburgh, PA) was used as the capture antibody and goat anti-mouse K conjugated to HRPO (Fisher Scientific) was the antibody used to detect the presence of the mouse K

chain. One of the human K positive, murine K negative
clones (HuK⁺, MuK⁻) was chosen to analyze antigen
specificity on MCF-7 cells and for expression levels by a
quantitative ELISA for human IgG expression on MCF-7
cells. A cell line that was antigen specific for MCF-7
cells and HuIgG⁺, MuIgG3⁻, HuK⁺, MuK⁻ was chosen and
designated Chimeric BR96 (Chi-BR96).

The original expression of the heavy and light
chain antigen specific chimeric BR96 (Chi-BR96) antibody
was approximately 25 µg/ml. Through four sequential
rounds of cloning the line in soft agarose with a rabbit
α HuIgG antibody overlay to detect cells secreting the
highest amount of chimeric antibody [Coffino et al., J.
Cell. Physiol. 79:429-440 (1972)], a hybridoma cell line
(ChiBR96) was obtained secreting approximately 130 µg/ml
of chimeric antibody . Hybridoma ChiBR96 was deposited
with the ATCC on May 23, 1990, and there provided with
the deposit number, ATCC No. HB 10460.

Binding of ChiBR96

The relative affinity of the ChiBR96 antibody
and murine BR96 antibody of the invention for the tumor
associated antigen on MCF-7 cells was determined by an
ELISA competition binding assay [Hellstrom et al., Cancer
Res. 50:2449-2454 (1990)]. Briefly, adherent antigen
bearing cell line MCF-7 was plated in a 96-well
microtiter dish at 3 X 10⁴ cells/well and allowed to grow
to confluency for about 3-4 days. The growth media was
discarded and the cells are fixed with 0.5%
glutaraldehyde in PBS (Sigma Chemical Co., St. Louis,
MO), at 100 µl/well for 30 min. The glutaraldehyde was
discarded and the plate was washed gently with PBS three
times. The plate was then blocked with binding buffer
(0.1% BSA in DMEM) 200 µl/well for 1 hr or was stored
indefinitely at -20°C. Binding buffer was discarded and

samples and standards were added to the wells. The
plates were covered and incubated overnight at 4°C.
Samples and standards were discarded and the plates were
washed three times with PBS. HRP-conjugate diluted in 1%
horse serum in PBS was added to wells, 100 μl/well and
incubated for 1 hr at 37°C. The ELISA was developed with
3,3',5,5'-tetramethyl-benzidine (TMB) chromagen (Genetic
Systems, Seattle, WA) in a citrate buffer. Color
development was arrested with 3N H_2SO_4 and the plate was
read on a Titertek Microplate reader at
450 nm. This assay determined how well 0.3 μg/ml of
biotinylated ChiBR96 antibody competes with either
unlabeled ChiBR96 or unlabeled murine BR96 monoclonal
antibody for the antigen. The bound biotinylated ChiBR96
antibody was detected with avidin-HRPO and developed with
standard ELISA reagents.

As shown in Figure 17, the overlap of the two
binding curves indicates that the two antibodies have the
same specificity and relative affinity for the tumor
antigen.

EXAMPLE 10

Characterization of the ChiBR96 Antibody and BR96 F(ab')₂
Fragments

Cytotoxicity of Unmodified ChiBR96 and BR96 F(ab')₂
Fragments

Living suspended cells from the BR96 antigen
positive carcinoma lines 3396, 2987 and MCF-7, were
treated with ChiBR96 and BR96 F(ab')₂ fragments prepared
as described in Examples 8 and 9, above, to determine
cytotoxicity of these antibodies as compared to the BR96
monoclonal antibody of the invention. The cytotoxicity
tests were performed by FACS assay as described above in

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Example 4. The results of these experiments are shown in
Figures 18-20 as percentage dead cells vs. antibody
concentration in µg/ml.

Figure 18 and 20 show that the chimeric BR96
antibody and F(ab')₂ fragments of BR96 IgG3 are similar to
BR96 monoclonal antibody with respect to cytotoxicity to
3396 and MCF-7 cells. Figure 19 demonstrates that the
cytotoxic effect on 2987 cells is much lower than on the
other breast carcinoma cells (Figures 18 and 20). These
results suggest that a higher binding ratio (Table 2) is
important for killing by these antibodies and/or that
different tumor cells might have different sensitivity to
killing by these antibodies. These results illustrate
that the ChiBR96 antibody and the F(ab')₂ fragments are
cytotoxic by themselves, i.e. in unconjugated form, and
also illustrate that the cytotoxicity of the BR96
antibodies is not dependent on the Fc region.

Internalization of ChiBR96

The internalization of the ChiBR96 antibody
within carcinoma cells was evaluated in comparison to
internalization of the BR96 monoclonal antibody. The
antibodies were conjugated to ricin A chain toxin to form
immunotoxins ChiBR96-RA (1-4 Ricin A chains per antibody
molecule) and BR96-RA (1-2 Ricin A chains per antibody
molecule) and internalization by carcinoma cell lines
3396 and 3630 was measured using a thymidine uptake
inhibition assay, as described in Example 3, above.

Graphs of the percent inhibition of thymidine
incorporation vs. immunotoxin concentration for each cell
line tested are shown in Figures 21 and 22. Figure 21
depicts the percent inhibition of thymidine incorporation
by cells from the 3396 breast carcinoma cell line caused
by internalization of ChiBR96-RA and BR96-RA. As shown

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in the graph, ChiBR96 is internalized similarly to BR96, and appears to be at least as efficient as BR96 at killing tumor cells. Similar results were obtained with the 3630 breast carcinoma cell line (Figure 22).

ADCC Activity of ChiBR96 Antibody

Determination of ADCC activity of ChiBR96 was conducted as described in Example 5, above using the following cell lines: breast cancer lines 3396, 3630 and 3680 (Oncogen, Seattle, WA) and MCF-7 (ATCC No. HTB22); ovarian cancer line 3633-3 (Oncogen, Seattle, WA); and lung cancer lines 2987; 3655-3 and 2981 (Oncogen, Seattle, WA). The results are shown in Table 3 for various antibody concentrations.

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TABLE 3
ADCC Activity of ChiBR96

Cell Lines	Antibody	NK	Antibody Concentration (µg/ml)				
			10	1	0.1	0.01	0.01
Breast Cancer 3396	BR96	28	86	74	58	27	25
	ChiBR96		88	79	60	34	26
MCF-7	BR96	16	82	69	54	17	15
	ChiBR96		90	82	57	25	17
MCF-7	BR96	22	73	69	48	22	22
	ChiBR96		76	70	57	33	26
3630	BR96	30	69	64	42	30	34
	ChiBR96		69	56	42	36	36
3680	BR96	13	73	67	58	34	38
	ChiBR96		70	71	61	39	30
Ovarian Cancer 3633-3	BR96	20	92	90	64	28	23
	ChiBR96		88	88	54	43	29
Lung Cancer 2987	BR96	11	51	57	41	9	7
	ChiBR96		69	65	51	28	15
3655-3	BR96	4	49	37	0	0	0
	ChiBR96		39	35	12	6	5
2981	BR96	3	4	3	3	4	5
	ChiBR96		5	4	3	4	4

The results shown in Table 3 for various antibody concentrations indicate that ChiBR96 mediates ADCC activity to a similar extent as BR96. The ADCC activity can be seen at antibody concentrations lower than those at which the ChiBR96 antibody is cytotoxic by itself. When antibody BR96 was used alone as a control it produced 0% killing at the concentrations tested. ADCC activity was only found with the BR96 antibody-binding cell lines.

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Ability of ChiBR96 to Mediate Complement-Mediated Cytotoxicity

Determination of the ability of ChiBR96 to kill tumor cells in the presence of human serum as a source of complement (CDC) were performed as described in Example 6, using breast cell lines 3396; MCF-7, 3630 and 3680; ovarian cancer cell line 3633-3; and lung cancer cell lines 3655-3, 2987 and 2981. Table 4 presents the results.

TABLE 4
CDC Activity of ChiBR96

Cell Lines	Antibody	Antibody Concentration (µg/ml)			
		10	1	0.1	0.01
Breast Cancer					
3396	BR96	100	99	78	13
	ChiBR96	86	92	13	2
MCF-7	BR96	94	100	63	2
	ChiBR96	92	83	1	0
3630	BR96	94	100	82	9
	ChiBR96	86	86	33	9
3680	BR96	100	100	19	7
	ChiBR96	87	100	5	9
Ovarian Cancer					
3633-3	BR96	98	98	21	0
	ChiBR96	100	100	26	1
Lung Cancer					
3655-3	BR96	91	22	0	0
	ChiBR96	46	3	0	0
2987	BR96	100	100	1	0
	ChiBR96	100	43	0	0
2981	BR96	0	3	3	2
	ChiBR96	1	1	2	10

As shown in Table 4, ChiBR96 gave a cytotoxic effect (CDC) similar to that of BR96, in the presence of human serum containing complement. BR96 and ChiBR96 were not cytotoxic in any concentration. Human serum was also not cytotoxic.

The above results demonstrate that the whole BR96 antibody and chimeric antibody of the invention are internalized within carcinoma cells to which they bind, are cytotoxic alone in unmodified form and have ADCC and CDC activity for cells expressing a higher amount of epitopes.

EXAMPLE 11

Evaluation of BR96 Antibodies In Vivo

The therapeutic potential of the unmodified BR96 antibody of the invention for treatment of tumors was examined in a series of experiments using human tumor xenografts in nude mice. In addition, certain parameters were examined that might influence the efficacy of BR96 as an antitumor agent. These parameters include level of antigen expression on the target tumor line, time from tumor implantation to initiation of therapy and effects of dose.

In all the _in vivo_ experiments, the required number of Balb/c nu/nu mice (Harlan Sprague Dawley, Indianapolis, IN) were implanted with either the human lung adenocarcinoma cell line H2987 or H2707 tumor line. Cells from these tumor lines were grown _in vitro_, harvested, washed and resuspended in PBS prior to subcutaneous (s.c.) implantation of 10 million cells into the rear flank of each mouse. These groups of mice were then randomized and separated into smaller equal groups of 8 or 10 mice each.

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To increase the chance of observing any antitumor effects of BR96 while still requiring the antibody to actually localize to the tumor implant site for any effect to occur, therapy was initiated 24 hours after tumor implantation on day 2. Both the BR96 and control MAbs were administered at the same dose and schedule, although initiation of therapy in some cases varied. The treatment dose was administered in 0.2 ml PBS intravenously (i.v.) through the tail vein of the mouse. Normally the schedule was once every three days for five injections (Q3DX5). However, two extra injections were given on days 19 and 21 after H2987 tumor implantation in the initial experiment.

Antitumor Effects of BR96 Antibody in 2987 and 2707 Tumors

Tumor volumes were determined for each animal weekly with measurement usually beginning on the eighth day after implantation. Tumor volumes were calculated from the measurements of tumor length and perpendicular width using the formula:

Tumor Volume = longest length X (perpendicular width squared/2)

Group mean values were then calculated and plotted against time after tumor implantation.

In the initial experiment depicted in Figure 23 treatment with BR96 resulted in highly significant anti-tumor effects against the H2987 cell line. BR64, which also binds and is internalized by these cells, was used as a negative control, and showed little if any effect compared to the PBS treated controls.

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Table 5 summarizes the effects on the individual tumors at the end of treatment in this first experiment.

TABLE 5

Effects of Treatment with Unmodified BR96 Initiated
At Different Times After H2987 Implantation

EXPERIMENT 1
DAY 28

		TUMOR RESPONSE			
GROUP	MAb	COMPLETE	PARTIAL	STABLE	PROGRESSION
1	BR96	2	0	3	5
2	BR64	0	0	1	9
3	PBS	0	0	0	10

Only treatment with BR96 antibody resulted in complete absence of tumor. Two animals in this group were tumor free and an additional 3 animals showed cessation of growth of their tumors following treatment with BR96 antibody. The two mice showing no signs of tumor remained tumor free throughout the course of the experiment.

Antitumor Effects of BR96 Antibody on Established Tumors

One of the ultimate goals of tumor therapy is the effective treatment of established and growing tumors. To examine whether BR96 could have an antitumor effect on established tumors the H2987 or H2707 lung adenocarcinoma tumor lines were used as xenografts in nude mice. Because both of these tumor lines result in palpable tumors eight days after administration of 10 million cells s.c., delaying initiation of treatment provided a method to examine antitumor effects on established tumors.

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Therefore, to further examine the efficacy of unmodified BR96, several experiments were performed where treatment was withheld for either 5 or 8 days following s.c. tumor implantation. The delay in treatment initiation allowed the tumor cells to become established tumors. This results in an animal model that is more difficult to treat but resembles the clinical situation in a more realistic manner.

The treatment protocol is summarized in Table 6. Three groups of 10 mice each were treated with BR96 antibody initiated at different times as described in this Table. Control mice received either FA6 or PBS beginning on DAY 2. FA6 is a murine IgG_3 directed against a bacterial antigen not found in mammalian species, and acted as an isotype matched nonbinding negative control monoclonal antibody.

TABLE 6

Effects of Treatment With Unmodified BR96 Initiated at Different Times After H2987 or H2707 Implantation

TREATMENT PROTOCOL

GROUP	MAb	SCHEDULE/ROUTE	DOSE	DAYS INJECTED
1	BR96	Q3DX5 i.v.	1 mg	2, 5, 8, 11, 14
2	BR96	Q3DX5 i.v.	1 mg	5, 8, 11, 14, 17
3	BR96	Q3DX5 i.v.	1 mg	8, 11, 14, 17, 20
4	FA6	Q3DX5 i.v.	1 mg	2, 5, 8, 11, 14
5	PBS	Q3DX5 i.v.	0.2 ml	2, 5, 8, 11, 14

The results of this treatment protocol for both H2987 and H2707 tumor cell lines are shown in Figure 24, where the number of animals without tumors versus when initiation of treatment after tumor implantation occurred are plotted. Absence of tumor, as defined by the absence

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of a palpable tumor, was assessed at the end of treatment
for each group. The day used for the determination of
tumor absence varied since treatment was initiated at
different times post tumor implant. Early initiation of
treatment was clearly more effective and efficacy
decreases as onset of treatment increased from time of
tumor implant. Since delay in initiation of treatment
allows greater growth and establishment of the tumor,
decreased efficacy at later treatment initiation times
reflects the increasing difficulty of treating larger and
more established tumors.

These results demonstrate that BR96 has
antitumor effects against two different tumor cell lines.
Antitumor effects were only observed in the three groups
treated with BR96 antibody while those animals treated
with either the control FA6 or PBS showed no antitumor
effects.

It is significant that the differences in
efficacy with more established tumors are greater with
the higher antigen expressing tumor line, H2707. The
observation that H2707 has a greater response to BR96
therapy than H2987 is consistent with the assumption that
the amount of antigen expressed by a tumor cell may
influence the efficacy of BR96 treatment. From the data
above it is clear that BR96 has antitumor effects against
staged tumors.

Dose Effects of BR96 Antibody

In another experiment, the dose effects of BR96
against the H2707 tumor line was examined. In this
experiment, BR96 was administered in decreasing half log
amounts from 1 mg/dose to 0.032 mg/dose. The mean tumor
volumes versus time post tumor implant of the groups are
presented in Figure 25. The control treated animals were

given only the highest dose of monoclonal antibody, 1
mg/dose BA6.   These control animals showed no antitumor
effects while there was a dose dependant response when
BR96 antibody was administered over the chosen dose
range.

Antitumor Effects of F(ab')₂ Fragment and Chimeric BR96

In addition, antitumor effects of the F(ab')₂
BR96 fragment were examined to determine if the antitumor
effects seen in vivo were due to the Fc portion or if
actual binding to the tumor with its subsequent
internalization was sufficient for the cell death, as
indicated by in vitro assays.   The dose of F(ab')₂
fragment was 0.66 mg/dose using the same schedule as the
whole BR96.   This dose corresponds to an approximate
molar equivalent of binding regions compared to the 1.0
mg/dose whole IgG₃ BR96.   Mean tumor volume values versus
time post tumor implantation for this group treated with
the antibody fragment are shown in Figure 26.   There were
clearly some antitumor effects although the effects were
not as strong as with whole antibody.   These effects were
most pronounced at the earlier time points during and
immediately following treatment.

Chimeric BR96 was also examined for antitumor
effects in this experiment.   An intermediate dose of 0.32
mg/dose for the chimeric Mab was chosen.   The mean tumor
volume values for this group of mice is also shown in
Figure 26.   Treatment with chimeric antibody BR96 was
more efficacious than a comparable dose of the murine
BR96 IgG₃.   This is further demonstrated in Figure 27
which shows that 6 of the 8 mice treated with chimeric
BR96 were free of palpable tumors at the end of
treatment.

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Examination of the individual tumors depicted
in Figure 27 shows that at completion of treatment a
clear dose effect was evident by the number of animals
without tumors after treatment with decreasing amounts of
whole IgG3 BR96 antibody from 1.0 to 0.1 mg/dose.
Surprisingly, treatment with 0.032 mg/dose resulted in an
antitumor effect similar to the 0.32 mg/dose. This may
reflect that the level of cells killed in the tumor from
the treatment was very close to the minimum amount
necessary for the tumor to continue to grow.

Three of the eight animals treated with the
F(ab')$_2$ fragment were free of palpable tumors after
treatment. Therefore the Fc portion is not entirely
necessary for the antibody to have antitumor effects _in
vivo_ although it should enhance the tumorcidal properties
of BR96, particularly in immunocompetent animals.

The above demonstrates that unmodified BR96
antibodies are effective antitumor agents against tumor
lines _in vivo_. Moreover, the BR96 antibodies have an
effect on staged or established growing tumors. There is
an indication that higher antigen density on the tumor
line may increase the ability of BR96 to kill these.
cells. It has been shown that any of the forms of the
monoclonal antibody, i.e., chimeric, murine whole IgG$_3$ or
F(ab')$_2$ fragments, are effective as antitumor agents.
Earlier treatment and higher doses are preferred.

EXAMPLE 12

Localization and Biodistribution of BR96 Antibodies

Radioiodinated BR96 monoclonal antibodies
administered at doses used in the therapy experiments
described above in Example 11, were used to determine
biodistribution characteristics. Specifically, the whole

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IgG, BR96, chimeric or F(ab')₂ fragments together with
the appropriate control (whole monoclonal antibody FA6,
chimeric 96.5 and 96.5 F(ab')₂, respectively) were used to
localize in the tumor and various other organs.

Prior to the localization experiments, animals
were injected with tumor cells as described above in
Example 11, for the therapy studies. However, the tumors
were allowed to grow in the animals for approximately 2
weeks. At this time, 100 μg of BR96 antibody or fragment
was radiolabeled with ^{125}I using 10 μg Iodogen for 10
minutes at room temperature as directed by the
manufacturer. Control antibody or fragments were labeled
with ^{131}I using the same method. These
radioiodinated antibodies were diluted with the
appropriate unlabeled antibodies to reach the doses used
in the therapy experiments. Both the specific and
nonspecific antibodies were then mixed and administered
together, i.v., through the tail vein of the mouse. At
selected times mice were randomly pulled from the group,
anesthetized, bled through the orbital plexus and
sacrified. Various tissues were removed, weighed and
counted on a gamma counter capable of differentiating
between the two radioisotopes of iodine. From this data,
percent injected dose and percent injected dose per gram
were calculated.

The accumulated data from the 24 post administration time
point in the localization experiments are summarized in
Table 7.

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TABLE 7

Summary of Biodistribution Experiments

% INJECTED DOSE/GRAM

24 HRS. POST ADMINISTRATION

	ANTIBODY	DOSE (mg)	TUMOR CELL LINE	BLOOD	TUMOR	LIVER	SPLEEN	KIDNEY	LUNG
1)	BR96-G₁	1.0	H2987	10.2	6.3	2.2	1.9	3.4	4.7
	FA6	1.0		6.3	2.1	2.1	1.6	2.4	3.2
2)	BR96-G₁	0.3	H2707	9.0	7.0	1.8	1.6	2.7	3.7
	FA6	0.3		5.9	2.7	2.0	1.8	2.2	2.8
3)	ChiBR96	0.32	H2707	7.2	8.2	1.4	1.6	2.0	3.5
	Chi96.5	0.32		7.5	2.3	1.8	1.6	1.9	3.5
4)	F(ab')₂								
	BR96	0.65	H2707	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3
	96.5	0.65		<0.3	<0.3	<0.3	<0.3	<0.3	<0.3

The only tissue showing significant differences between specific and nonspecific antibody is the tumor. All other tissues examined show approximately equal uptake between the specific and nonspecific antibodies. One possible exception is the lower blood levels for the nonspecific antibody, FA6. This indicates accelerated blood clearance of this antibody. However, the difference between the specific and nonspecific antibody in the tumor is greater than the difference in blood levels between the FA6 and BR96 antibodies.

The data in Table 7 also demonstrate that the percent of the dose present in a particular organ is constant regardless of the dose administered. This would therefore indicate there is quantitatively more antibody present at the tumor site when higher doses are administered. In addition, there are no apparent differences between the two tumor lines with respect to specific vs nonspecific uptake.

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Table 7 also demonstrates that the F(ab')$_2$ was cleared from the animal at a much faster rate than either the IgG$_3$ or chimeric BR96. This could explain the reduction in efficacy of the fragment compared to the whole antibody therapy experiments. Any antitumor effects from the fragment must therefore be rapid and occur during the short time span prior to being cleared.

ChiBR96 localized at a comparable level to the IgG$_3$ BR96. Higher amounts were present only in the tumor compared to the control chimeric antibody. This suggests that any increase in efficacy of the chimeric antibody compared to the murine BR96 IgG$_3$ is due to the human constant region substitution. Of equal importance, the human constant region substitution does not appear to effect the ability of the chimeric antibody to localize to the tumor or adversely affect its biodistribution.

In summary, the IgG$_3$ and chimeric forms of BR96 are capable of specifically localizing to the tumor site. Moreover, both localization and therapeutic effects have been shown in these preliminary experiments at comparable doses. Indirect evidence of localization of the F(ab')$_2$ fragments was shown by the antitumor activity of the fragments in the therapy experiments. This activity must occur before 24 hours.

While we have presented particular embodiments of this invention herein, it is apparent that variations and modifications can be effected within the scope of the invention. Therefore, it will be appreciated that the scope of this invention is to be defined by the claims appended hereto rather than by the specific embodiments which have been presented herein by way of example.

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CLAIMS

We claim:

1. An antibody or fragment thereof that has specific immunological reactivity with human carcinoma cells, said antibody characterized by internalization within the carcinoma cells with which it reacts, mediation of ADCC and CDC activity, and killing of said human carcinoma cells in the absence of host effector cells or complement.

2. The antibody of claim 1, wherein the antibody is a murine monoclonal antibody.

3. Murine monoclonal antibody BR96 produced by the hybridoma having the identifying characteristics of HB 10036 as deposited with the ATCC.

4. Hybridoma HB 10036 as deposited with the ATCC.

5. A recombinant protein comprising the antigen-binding region of the monoclonal antibody of claim 2.

6. The recombinant protein of claim 5, wherein the protein is a human/murine chimeric antibody.

7. Chimeric antibody ChiBR96 produced by the hybridoma having the identifying characteristics of HB 10460 as deposited with the ATCC.

8. Hybridoma HB 10460 as deposited with the ATCC.

9. The recombinant protein of claim 5, wherein the antigen-binding region is joined to at least a functionally active portion of a second protein having anti-tumor activity.

10. The recombinant protein of claim 9, wherein the second protein is an enzyme, lymphokine, oncostatin or toxin.

11. The recombinant protein of claim 5, wherein the antigen-binding region reacts with an antigen that is a variant of Le^y antigen including an epitope containing fucose α1-3.

12. An Fab, F(ab')$_2$ or Fv fragment of the antibody of claim 2.

13. A bispecific antibody with a binding specificity for two different antigens, one of the antigens being that with which the monoclonal antibody of claim 2 binds.

14. The bispecific antibody of claim 13, wherein one of the antigens is a variant of Le^y antigen including an epitope containing fucose α1-3.

15. A monoclonal antibody, the antigen-binding region of which competitively inhibits the immunospecific binding of monoclonal antibody BR96 produced by hybridoma HB 10036 to its target antigen.

16. The antibody of claim 15 wherein said antigen is a variant of Le^y antigen including an epitope containing fucose α1-3.

17. A human/murine recombinant antibody, the antigen-binding region of which competitively inhibits the immunospecific binding of monoclonal antibody BR96 produced by hybridoma HB 10036 to its target antigen.

18. The antibody of claim 3 or 7 wherein the antibody is conjugated to a therapeutic agent to form an antibody conjugate.

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19.   The antibody of claim 18 wherein the therapeutic
agent is an anti-tumor drug, a toxin, a radioactive
agent, a second antibody or an enzyme.

20.   A combination of an immunoconjugate comprising the
antibody of claim 3 or 7 linked to an enzyme capable of
converting a prodrug into a cytotoxic drug, and said
prodrug.

21.   A pharmaceutical composition useful in the treatment
of human carcinomas comprising a pharmaceutically
effective amount of the antibody of claim 3 or 7.

22.   A pharmaceutical composition useful in the treatment
of human carcinomas comprising a pharmaceutically
effective amount of at least one antibody conjugate
according to claim 18.

23.   A pharmaceutical composition useful in the treatment
of human carcinomas comprising a pharmaceutically
effective amount of at least one recombinant protein
according to claim 9.

24.   A method of treating human carcinomas <u>in vivo</u>
comprising administering to a patient a pharmaceutically
effective amount of a composition containing the antibody
of claim 3 or 7.

25.   A method for determining the presence of carcinoma
in human tissue comprising contacting a specimen of said
tissue with the antibody of claim 3 or 7 and detecting
the binding of said antibody to said tissue.

26.   The method of claim 25 wherein said antibody is
labeled so as to produce a detectable signal with a label
selected from the group consisting of a radiolabel, an
enzyme, a chromophore and a fluorescer.

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27. A method for imaging human carcinoma comprising administering to a patient the antibody of claim 3 or 7 in an amount effective for detection of the antibody intravenously, allowing the antibody to localize to the site of carcinoma cells and detecting said antibody.

28. The method of claim 27 wherein said antibody is labeled so as to produce a detectable signal with a label selected from the group consisting of a radiolabel, an enzyme, a chromophore, and a fluorescer.

29. A monoclonal anti-idiotypic antibody reactive with an idiotype on the antibody of claim 3.

30. A diagnostic kit comprising:

     a) the antibody of claim 3 or 7; and

     b) a conjugate of a detectable label and a specific binding partner of the antibody of

          (a) above.

31. The diagnostic kit of claim 30 wherein the label is selected from the group consisting of enzymes, radiolabels, chromophores and fluorescers.
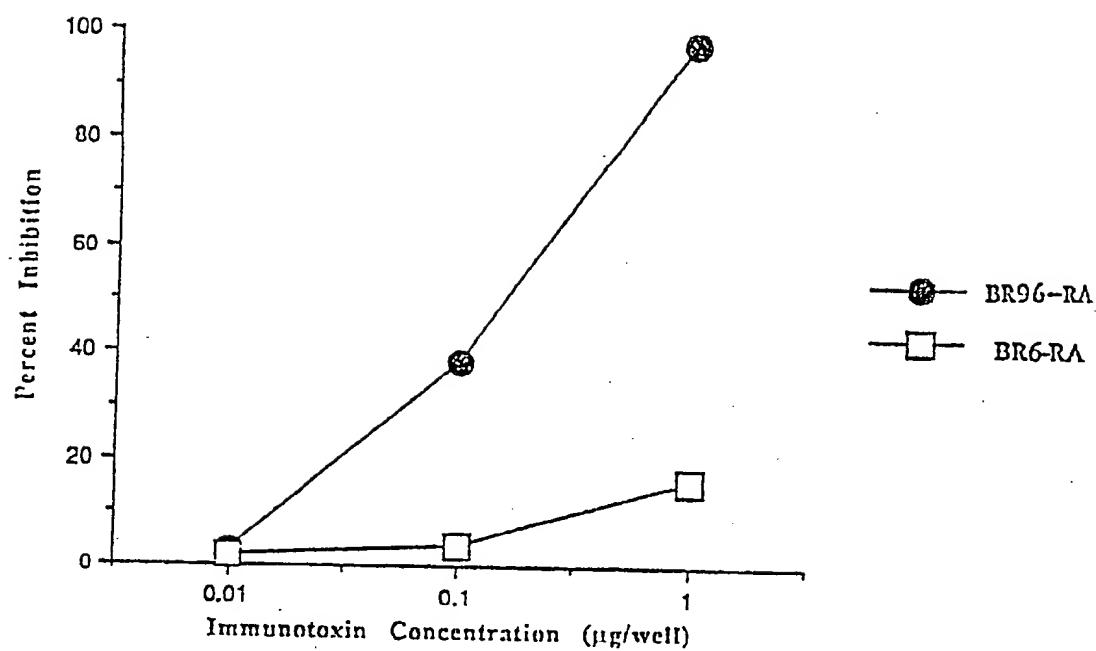
FIGURE 1

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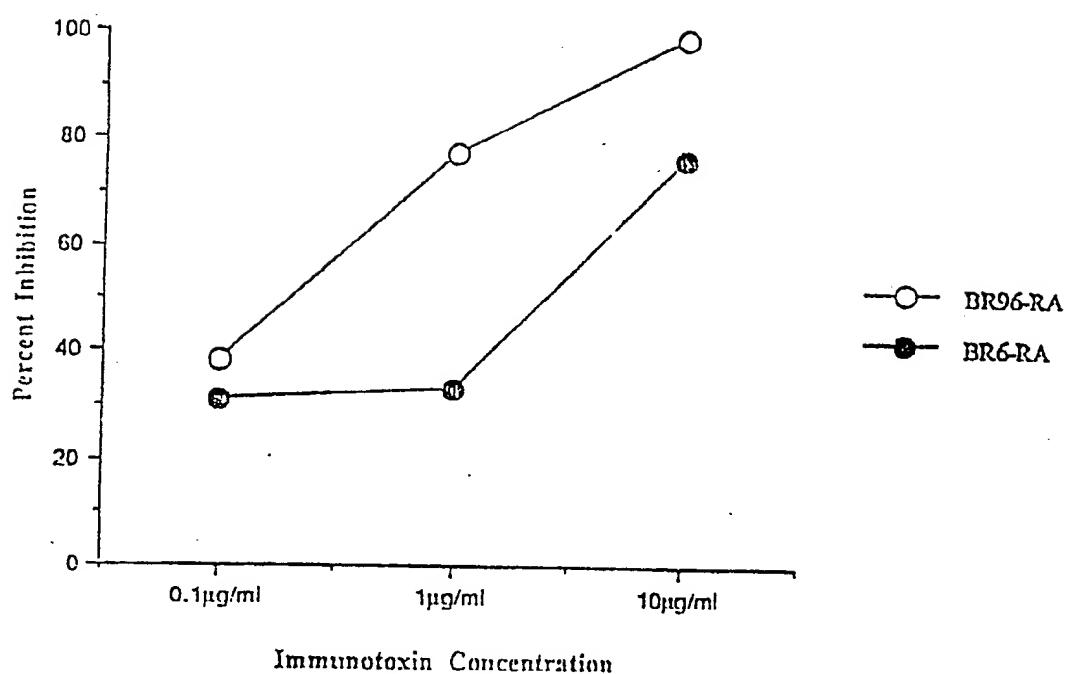
FIGURE 2

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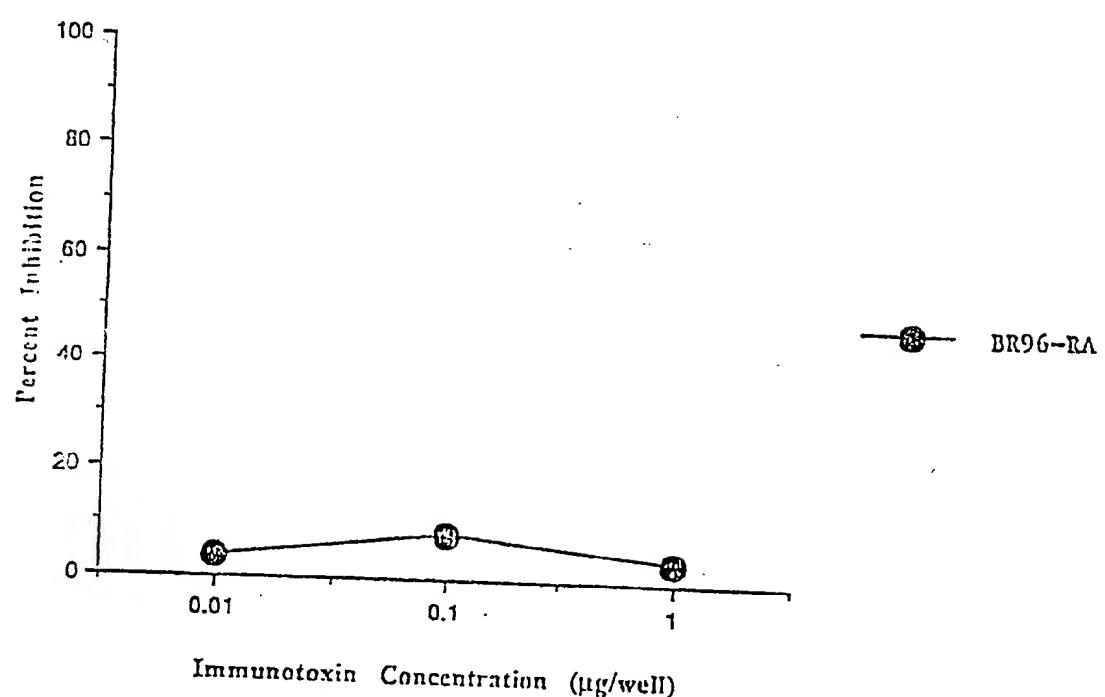
FIGURE 3
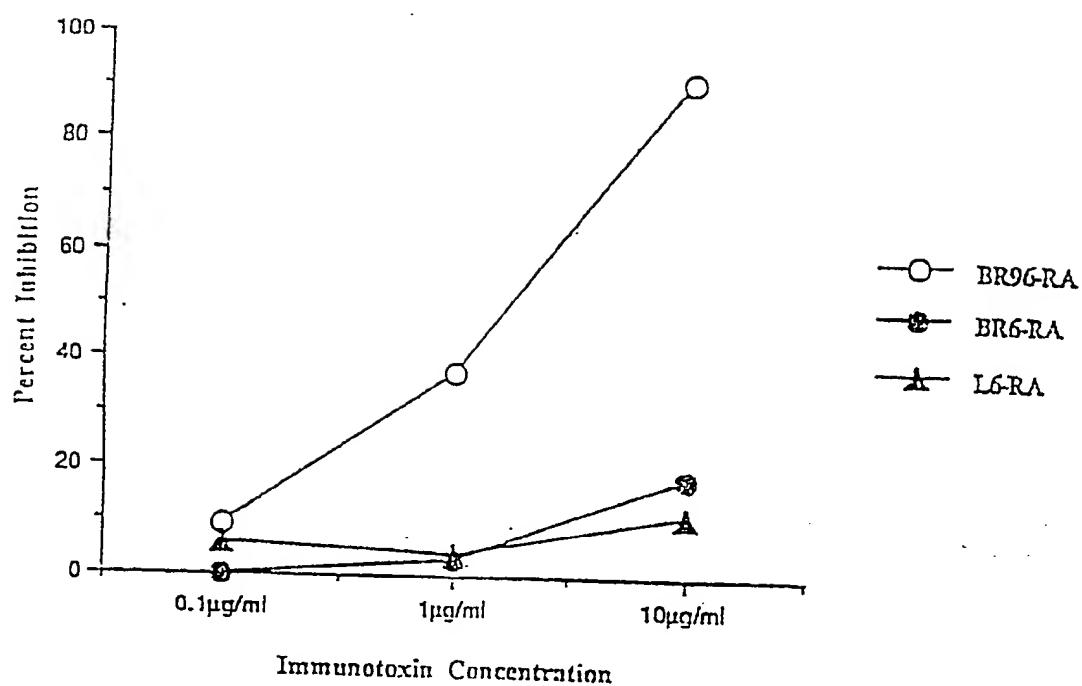
FIGURE 4

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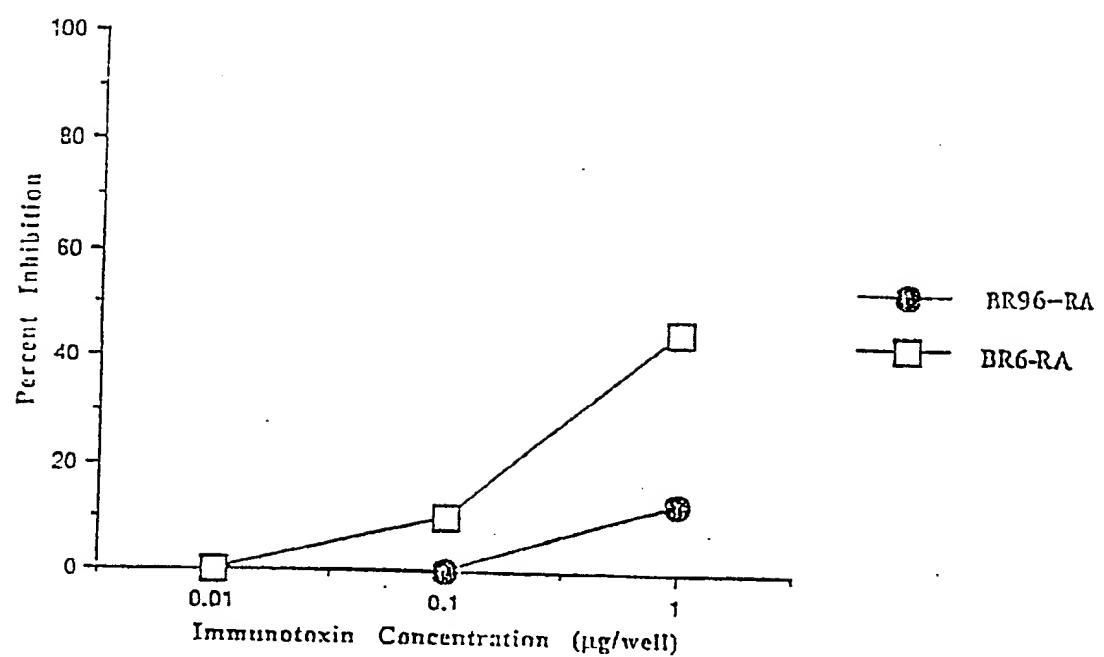
FIGURE 5
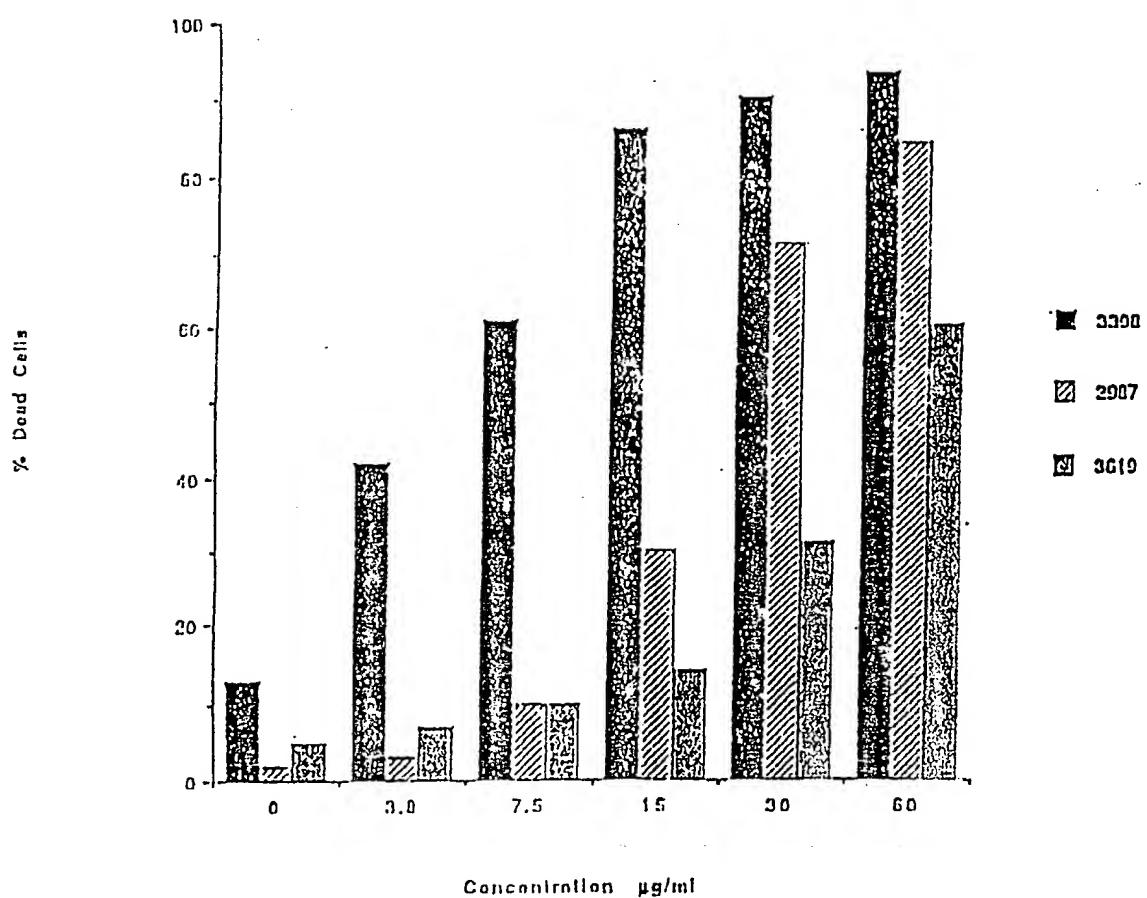
FIGURE 6

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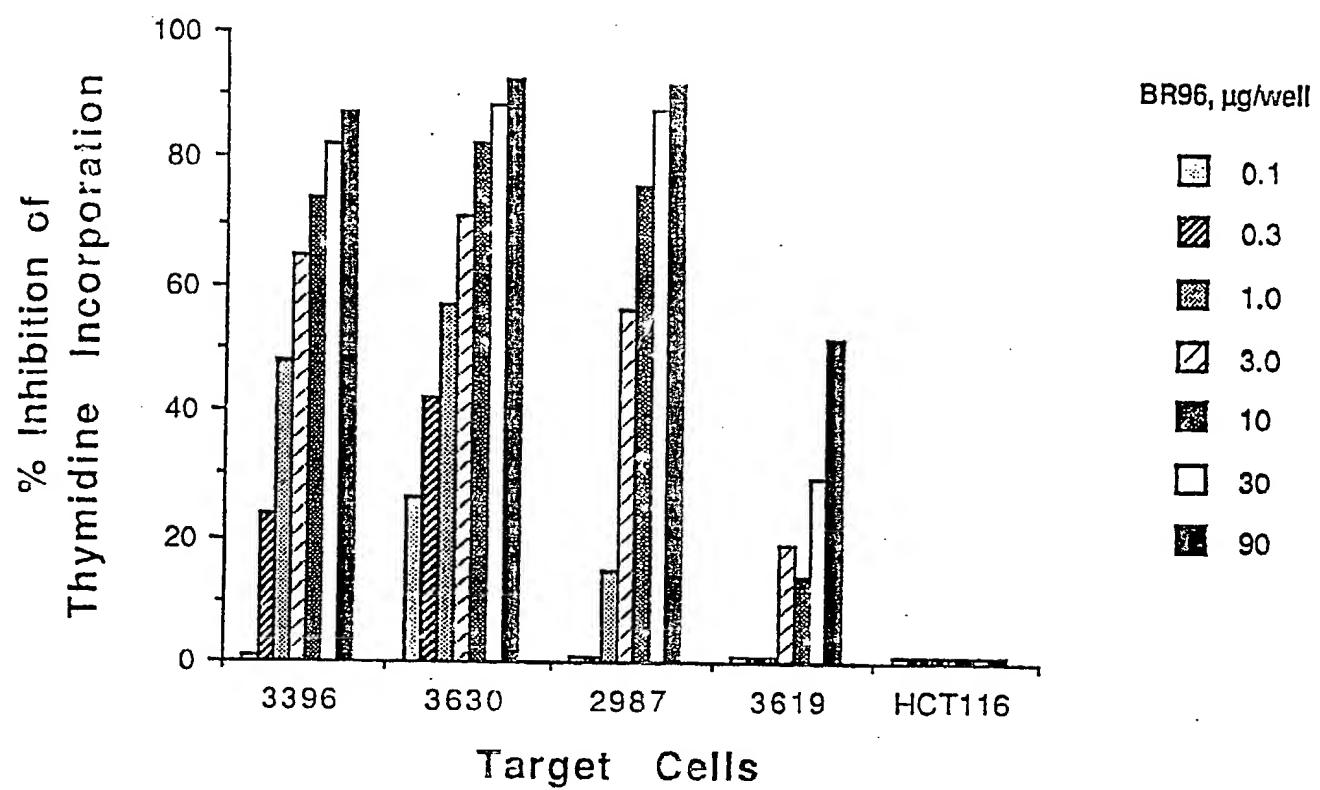
FIGURE 7
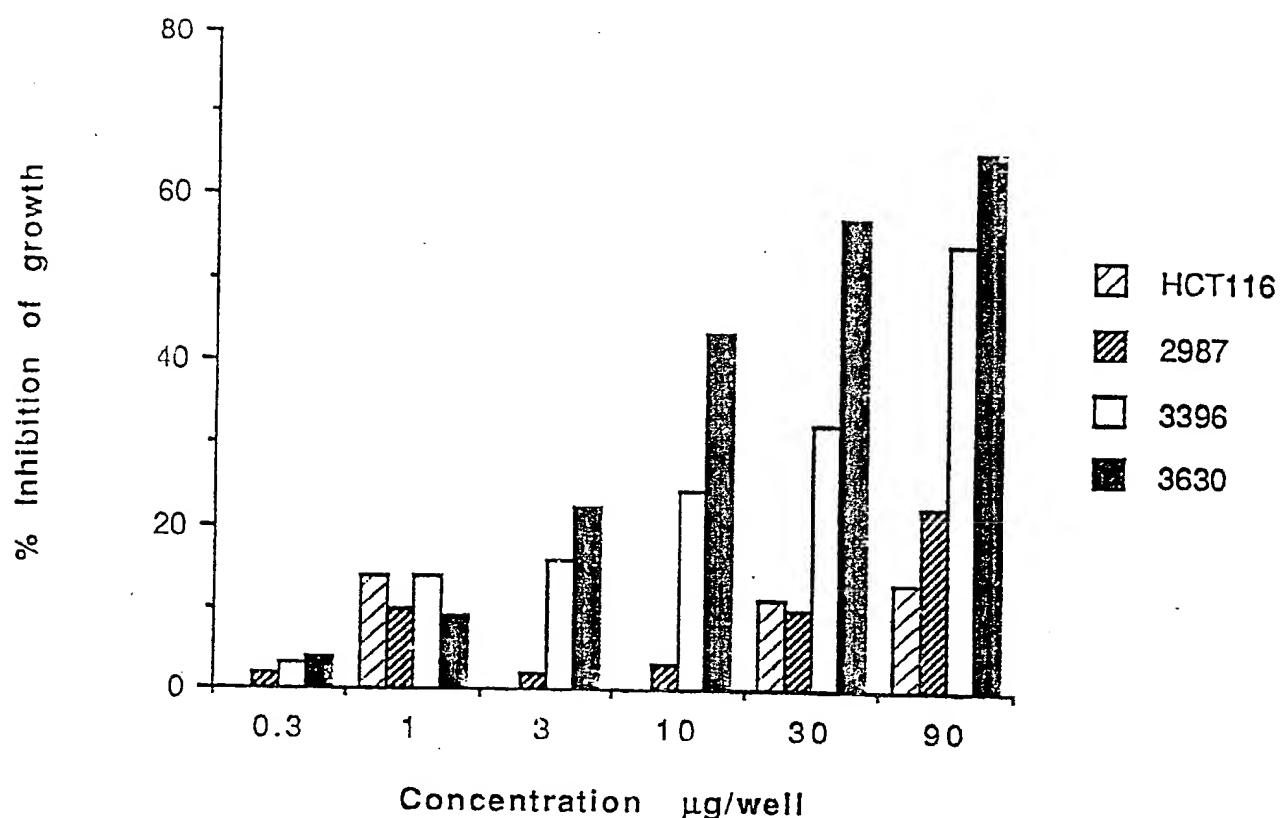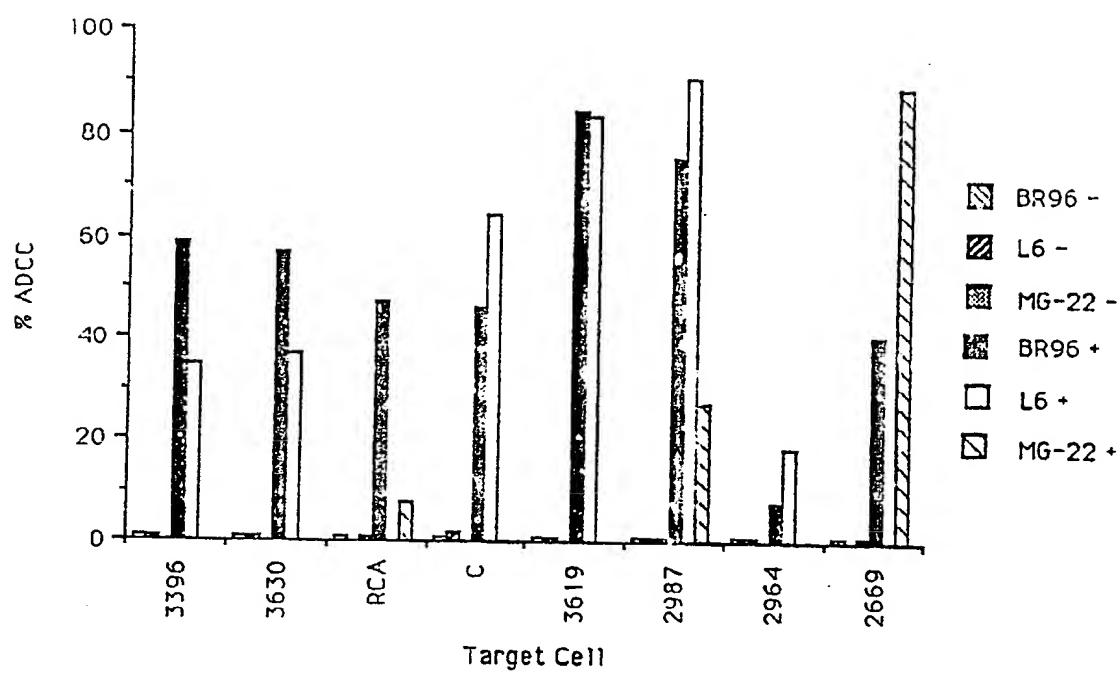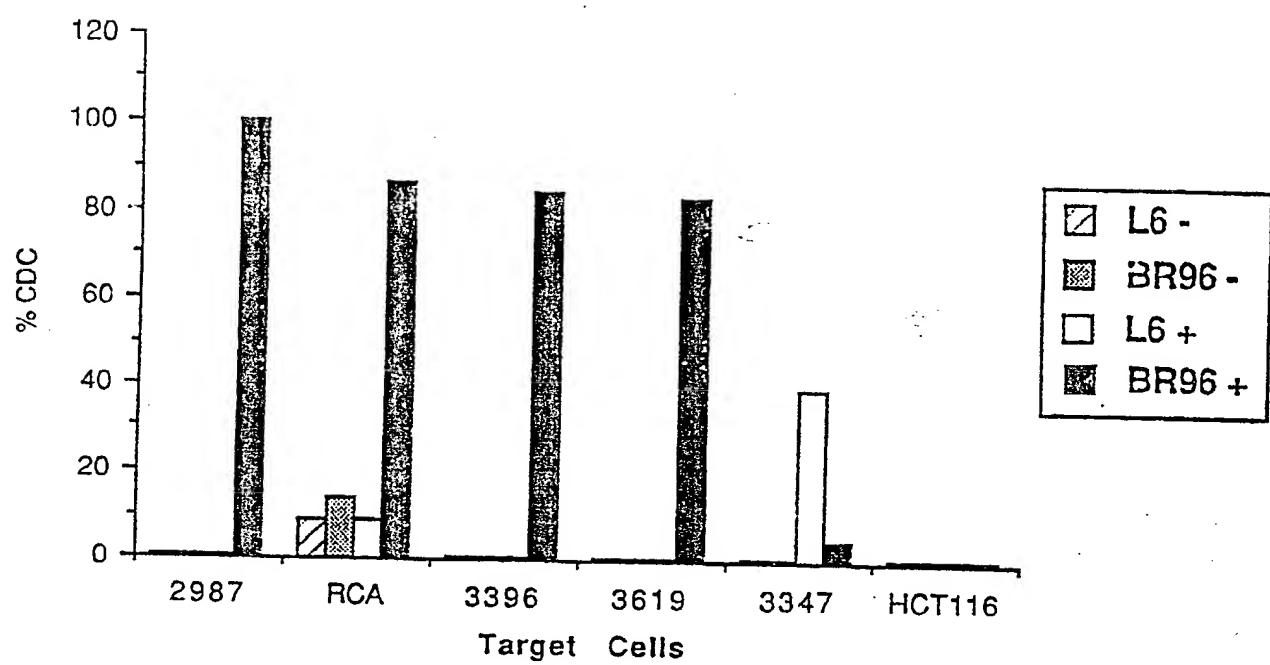
FIGURE 8

FIGURE 9

FIGURE 10

Absorbance at 450nm

FIGURE 11

Absorbance at 450nm

LNFI	0.203
LNFII	0.199
LNFIII	1.47
LNDI	0.18
Maltoso	0.211
Lactose	0.147
LNT	0.203
LNnT	0.181
LNH	0.25
LNnH	0.129
Molibioso	0.159
Collobioso	0.137
A-tri	0.184
B-tri	0.174
A-Tatrose	0.206
2'-Fuco	0.159
A Hopta	0.117
Ganglio	0.122
T antigon	0.241
3'-SL	0.148
6'-SL	0.155
LSTa	0.135
LSTb	0.143
LSTc	0.131
S Le a	0.278
Disialyl	0.124
Chitotrioso	0.181
Man 1-3M	0.147
Man 1-2M	0.119
Biant octa	0.107
Globotri	0.177
Globotet	0.134
→ Le y	≥2
GM1'Octa	0.159
S Le x	0.117
Blank	0.16
Pos Control	0.09
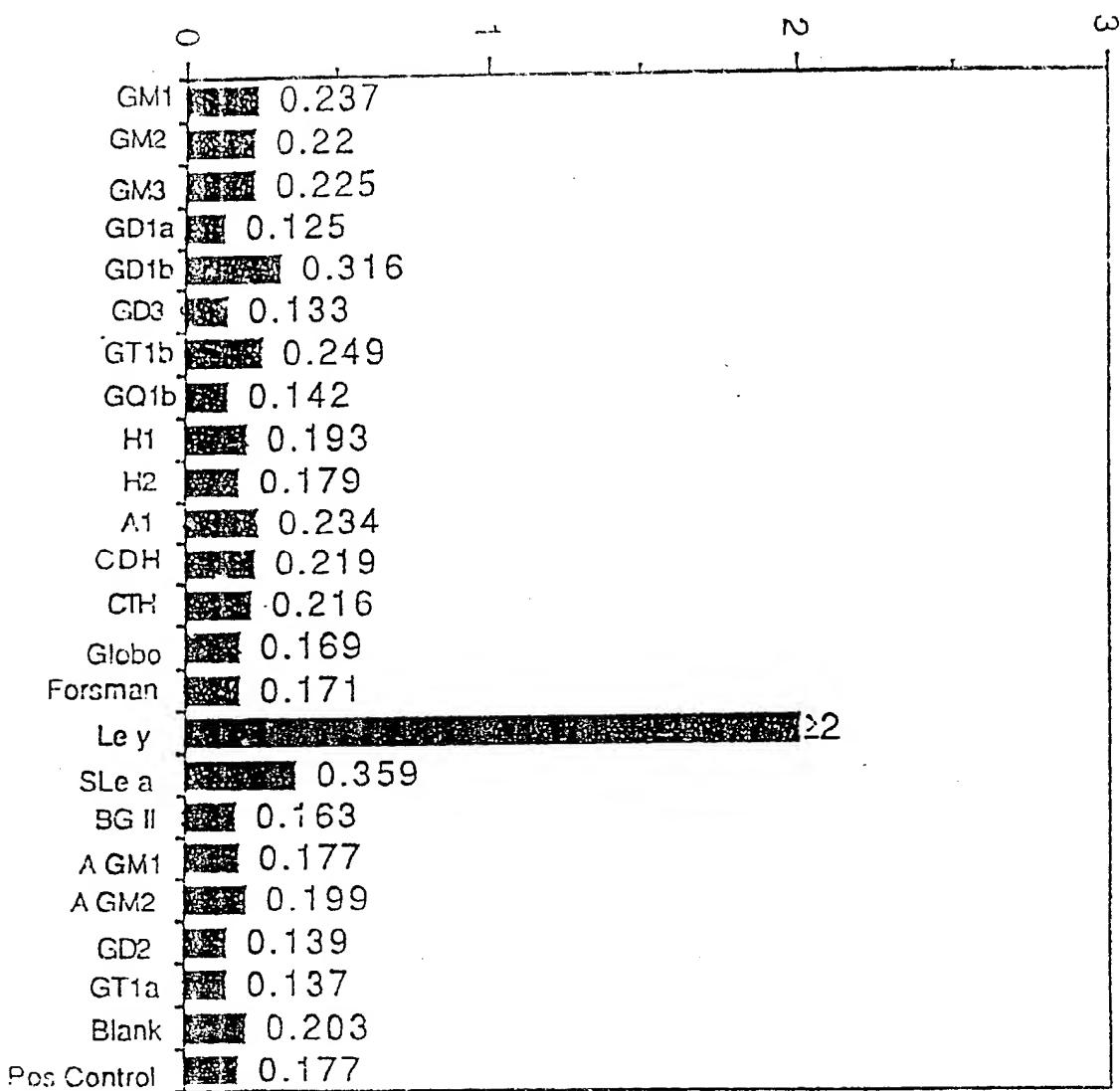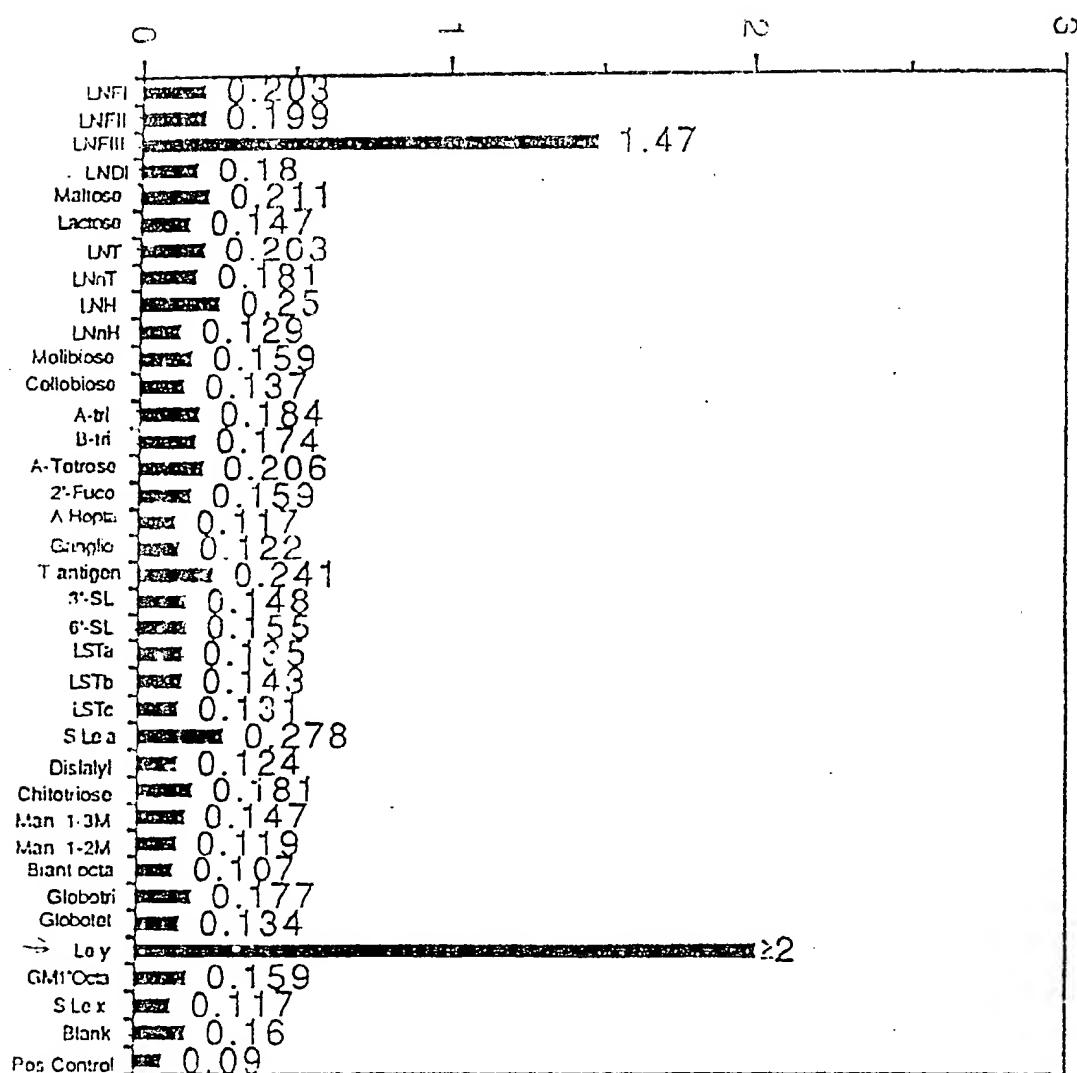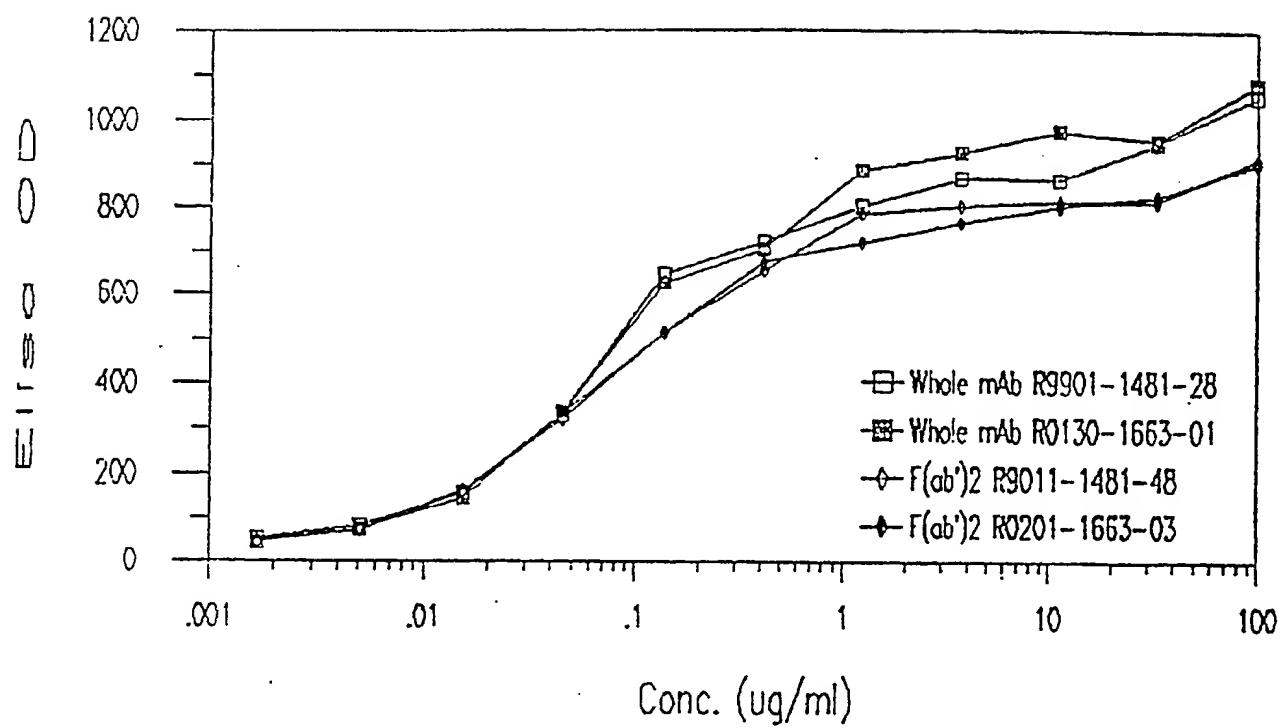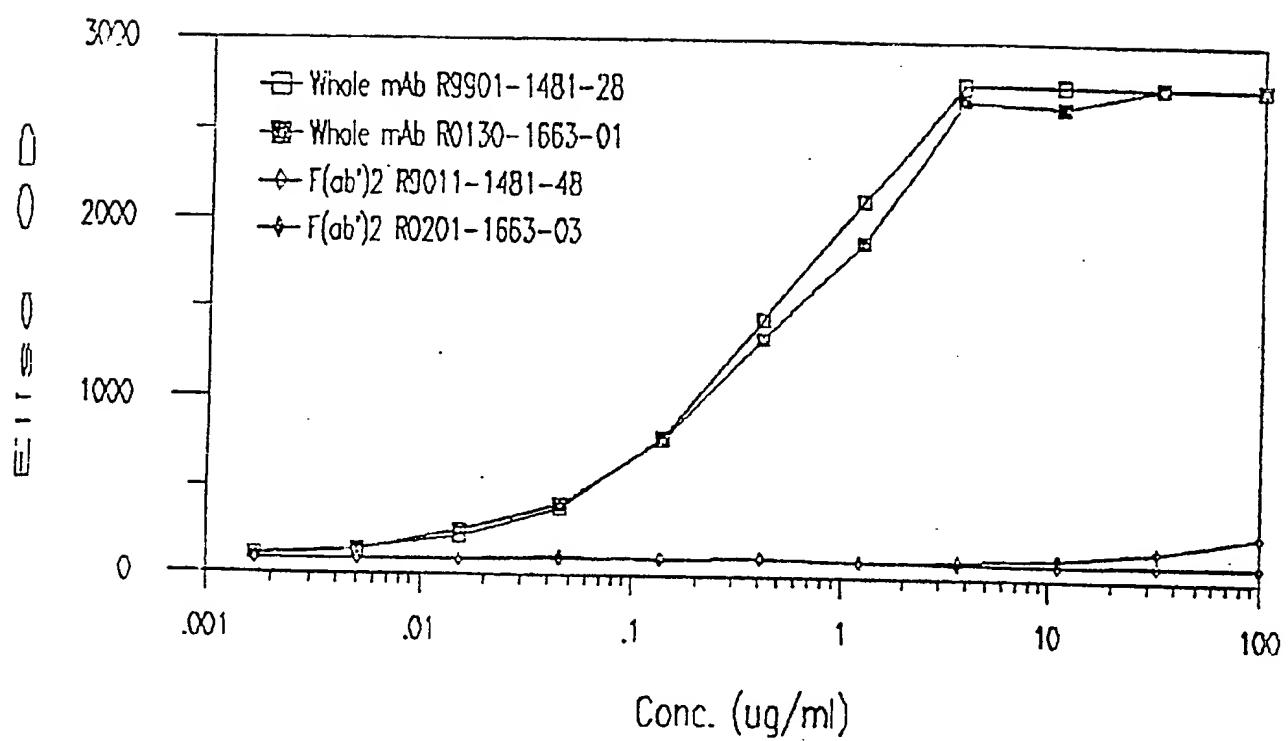
FIGURE 12

FIGURE 13

FIGURE 14

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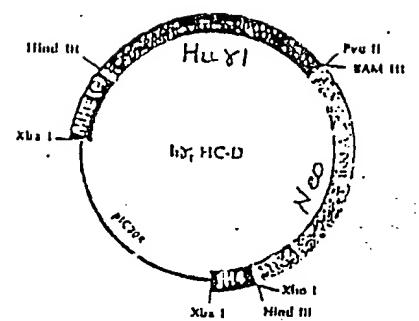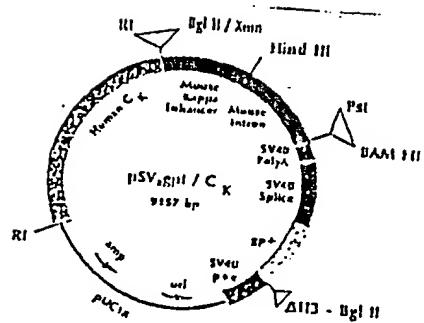
FIGURE 15

FIGURE 16

Characterization of Chimeric Antibody Specificity and Affinity by a Competition Binding Assay

Competition Binding Assay
0.3 µg/ml Biotinylated Chimeric BR96
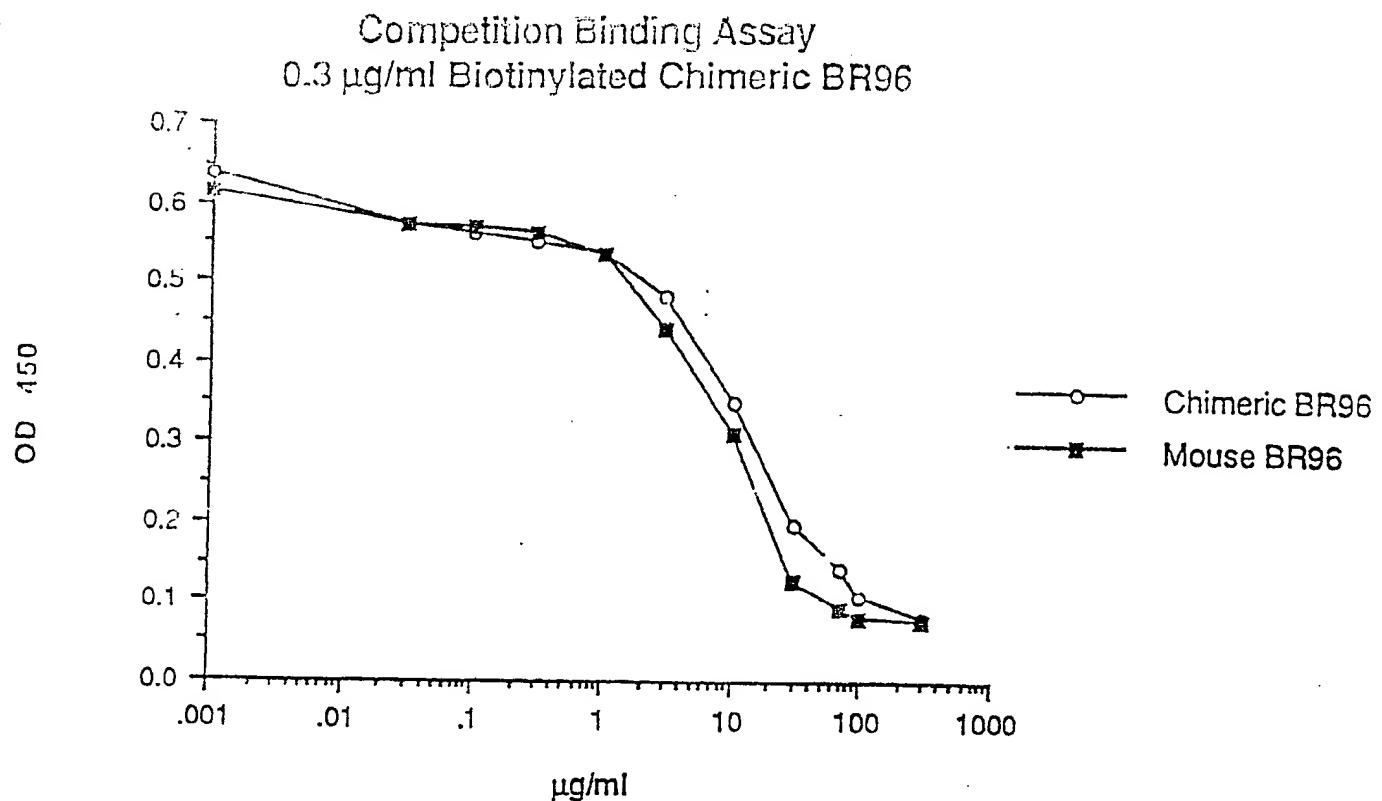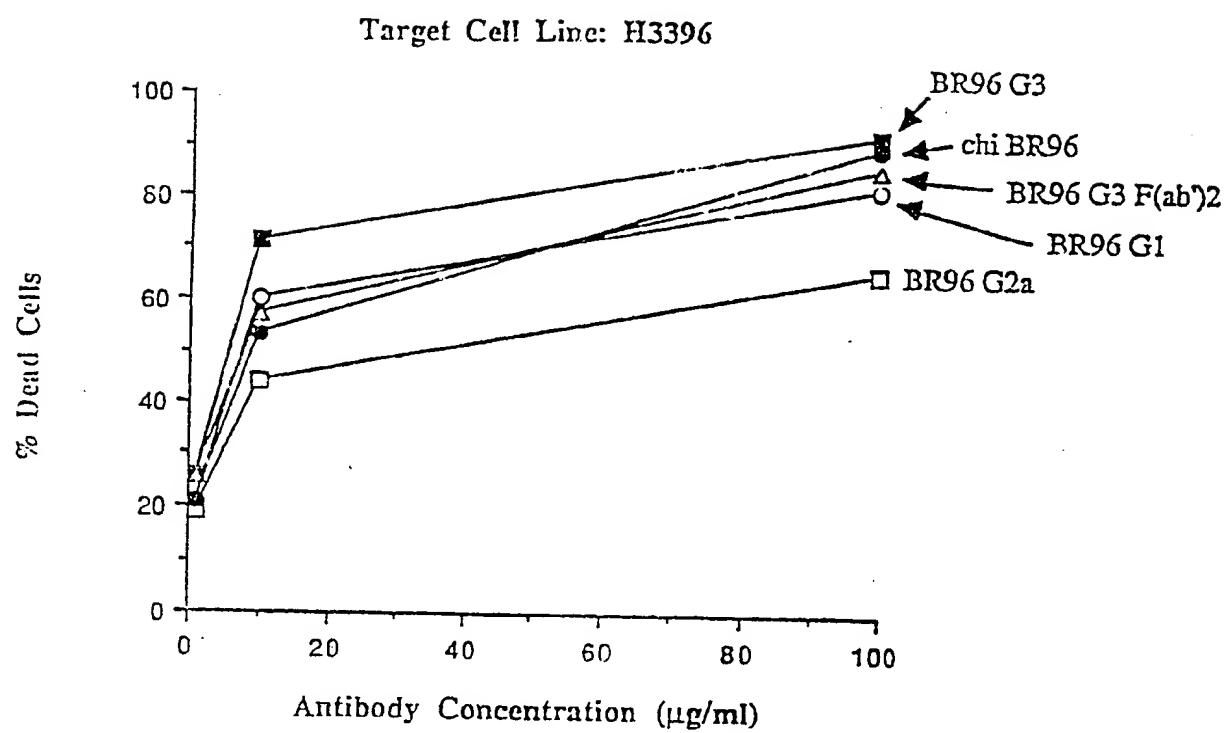
FIGURE 17

Target Cell Line: H3396

FIGURE 18

Target Cell Line: H2987
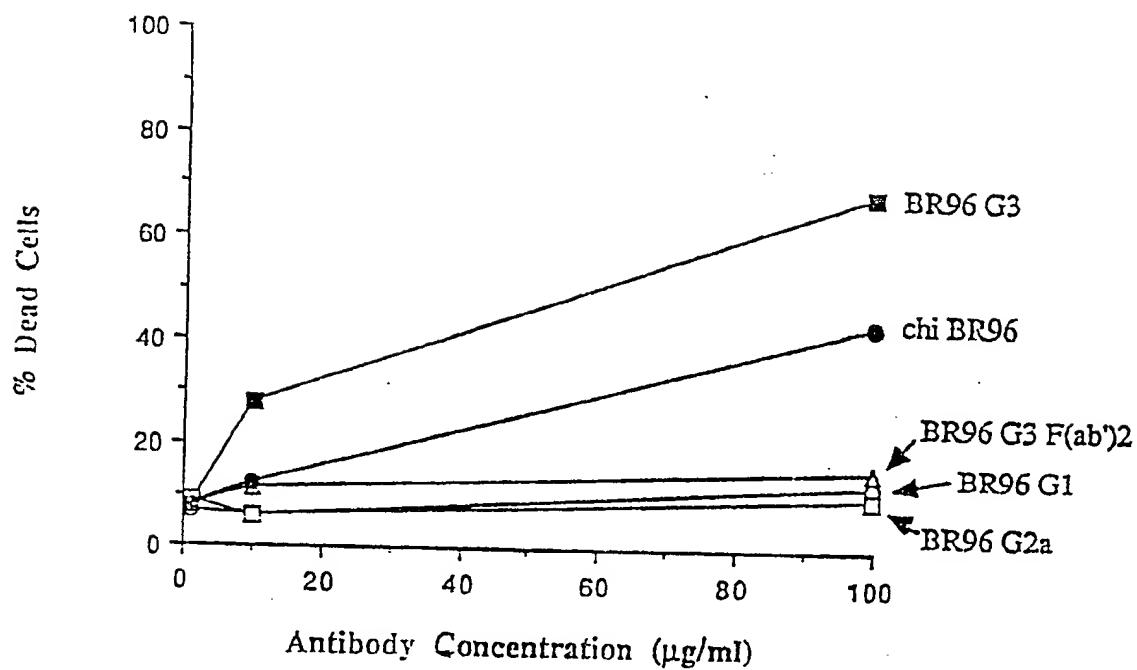
FIGURE 19
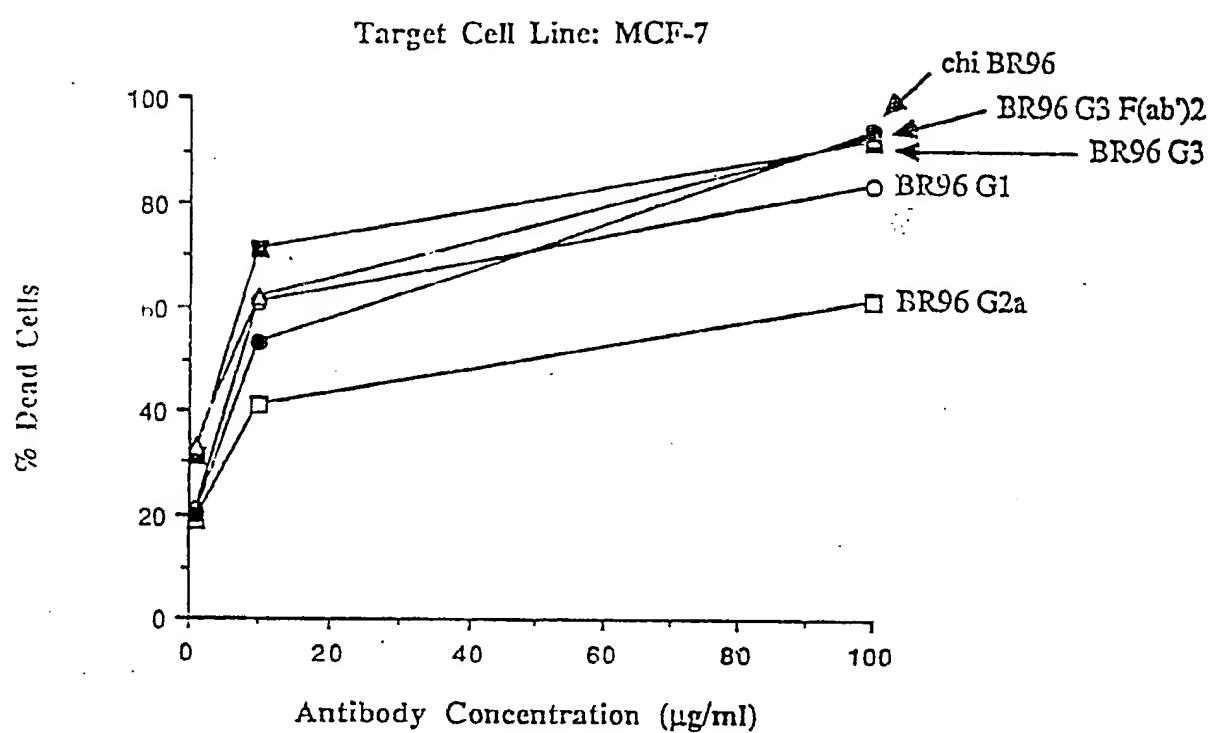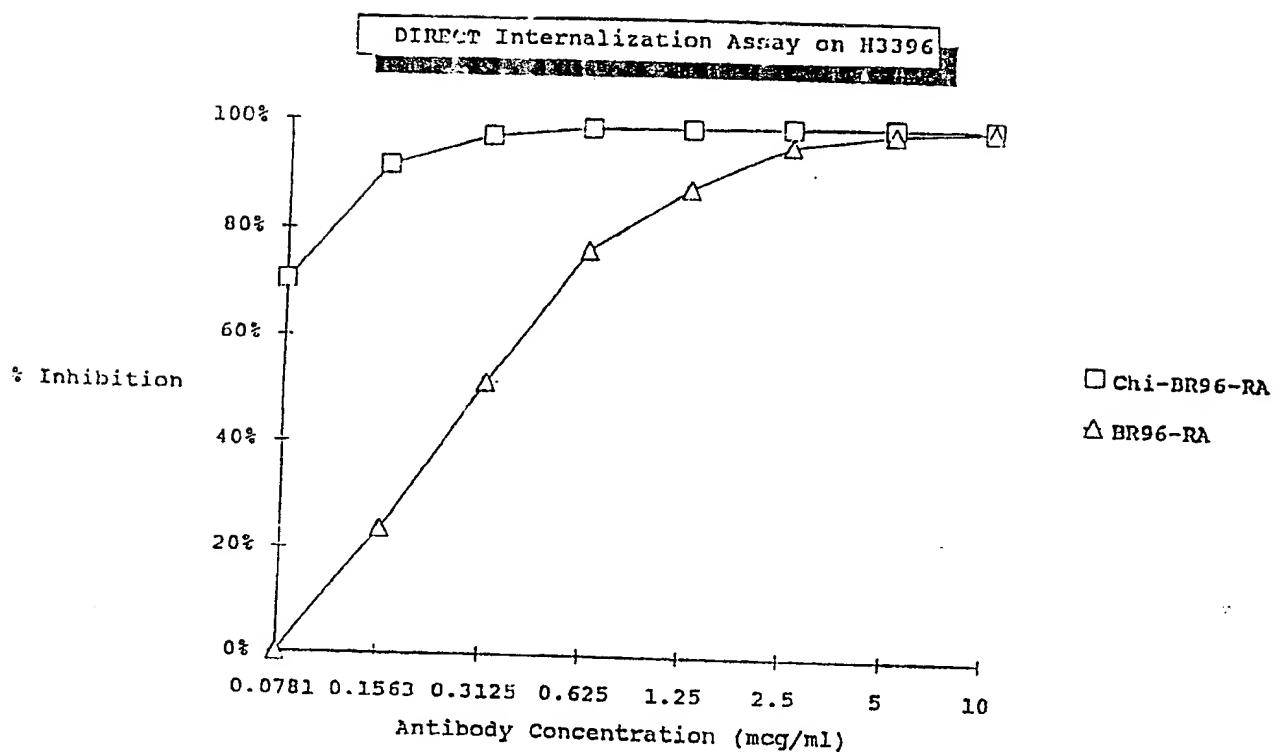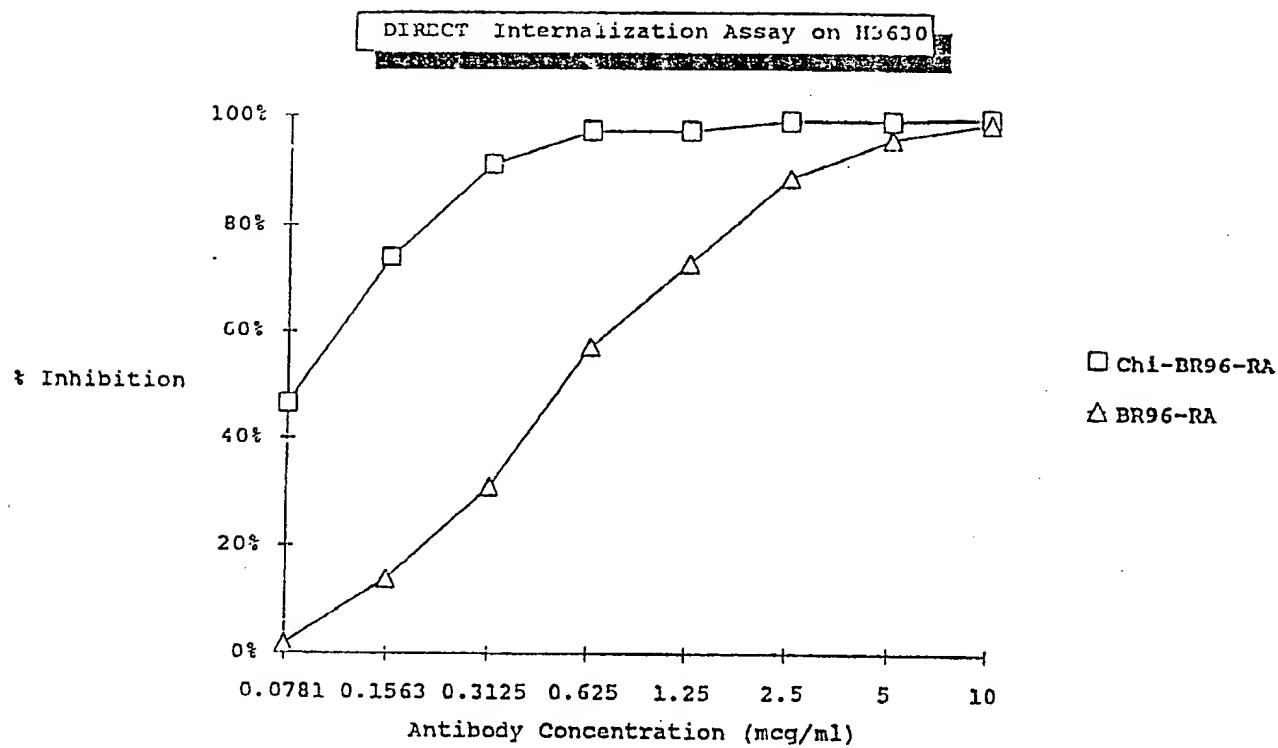
FIGURE 20

FIGURE 21

FIGURE 22

EFFECTS OF UNMODIFIED MAbs ON H2987
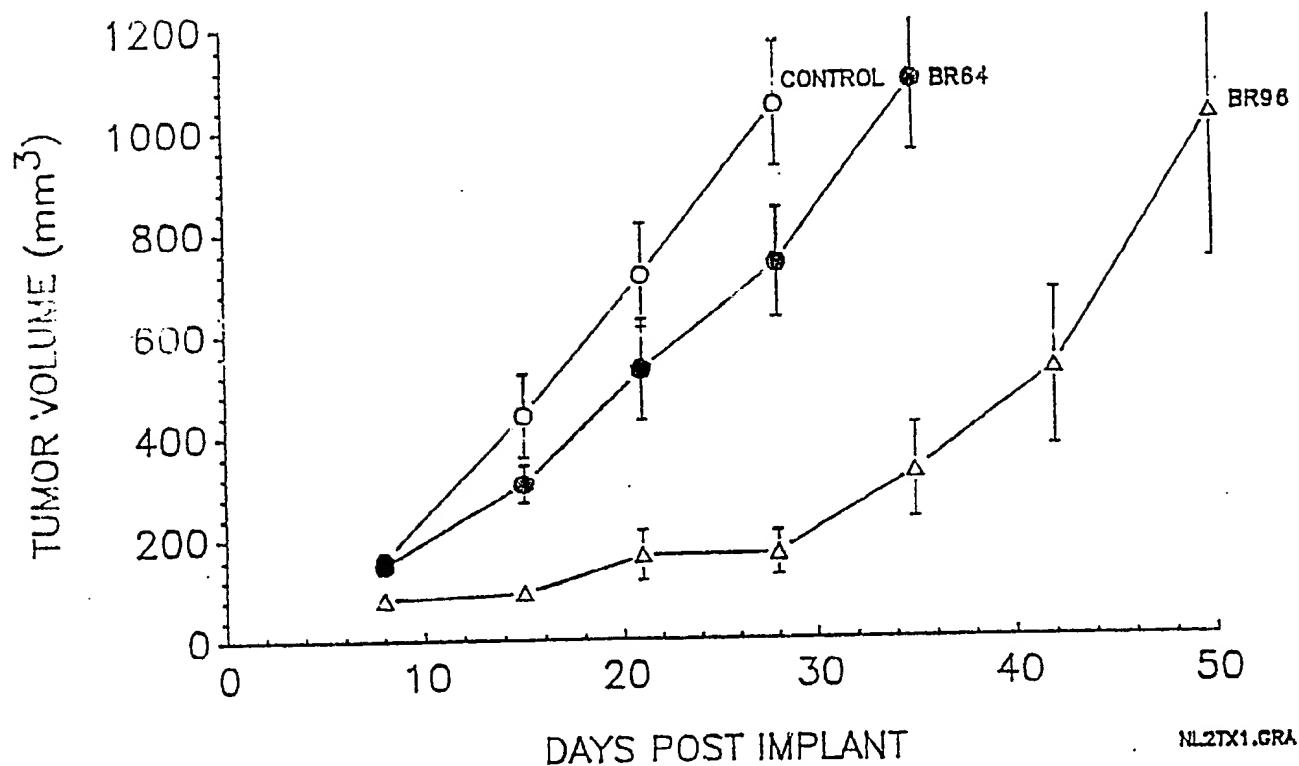
FIGURE 23

# ABSENCE OF TUMOR AT END OF TREATMENT
10 ANIMALS PER GROUP
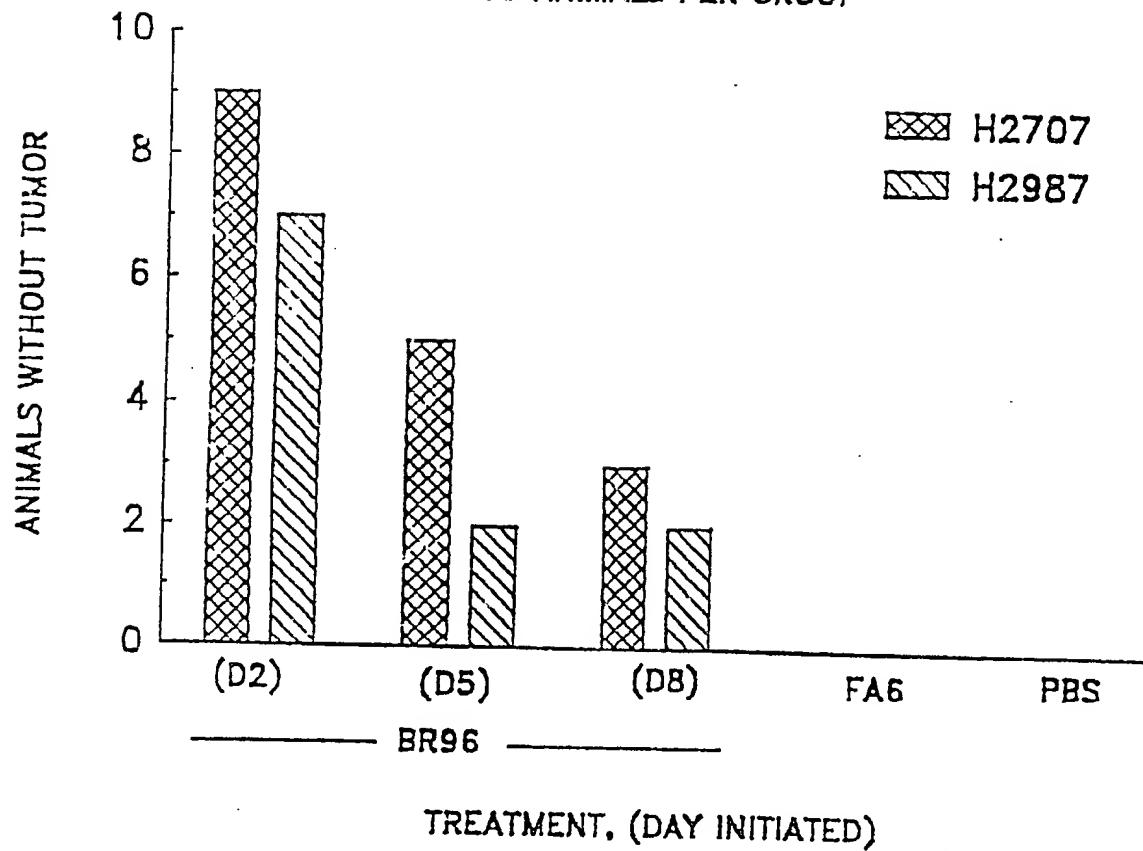
FIGURE 24

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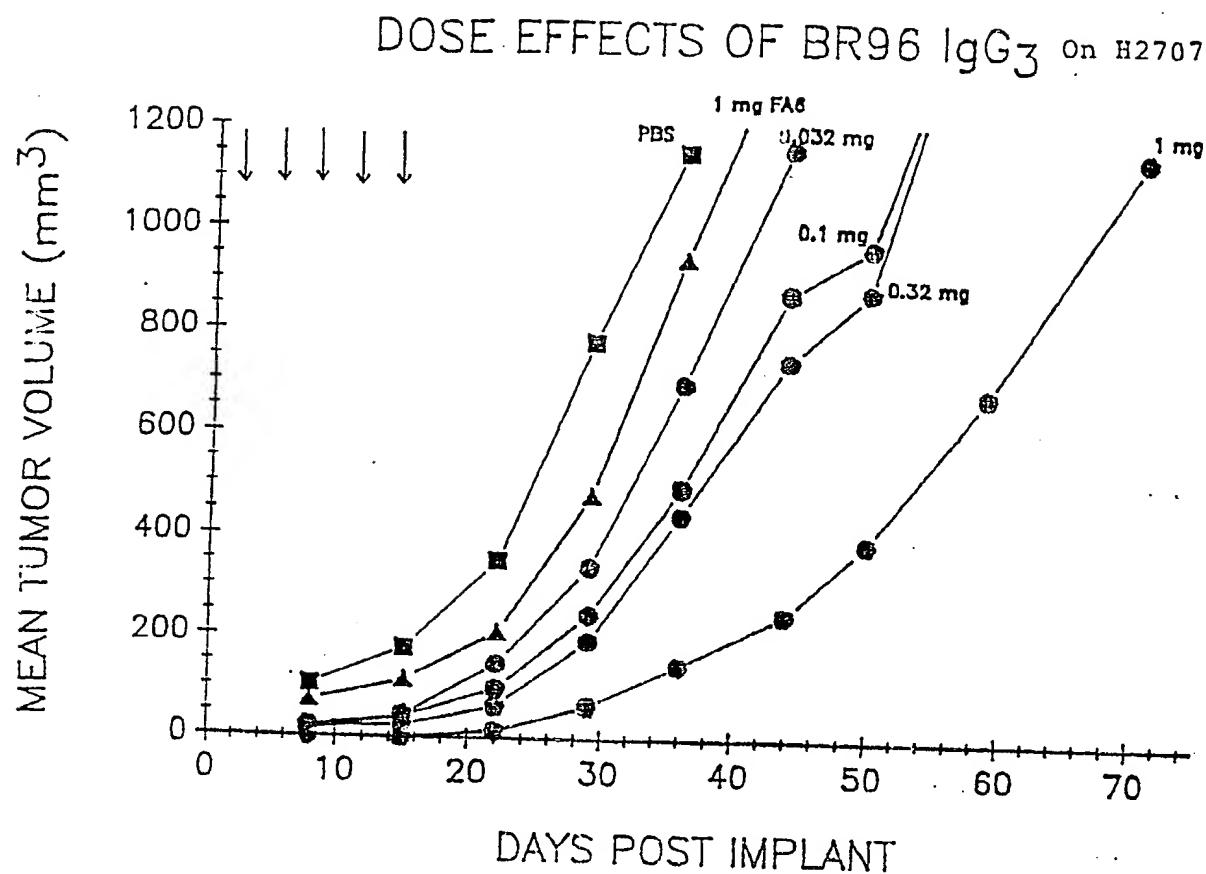
FIGURE 25

EFFECTS OF F(ab')$_2$ AND CHIMERIC BR96 ON H2707

FIGURE 26

## ABSENCE OF TUMOR AFTER TREATMENT
8 ANIMALS PER GROUP
H2707 XENOGRAFT
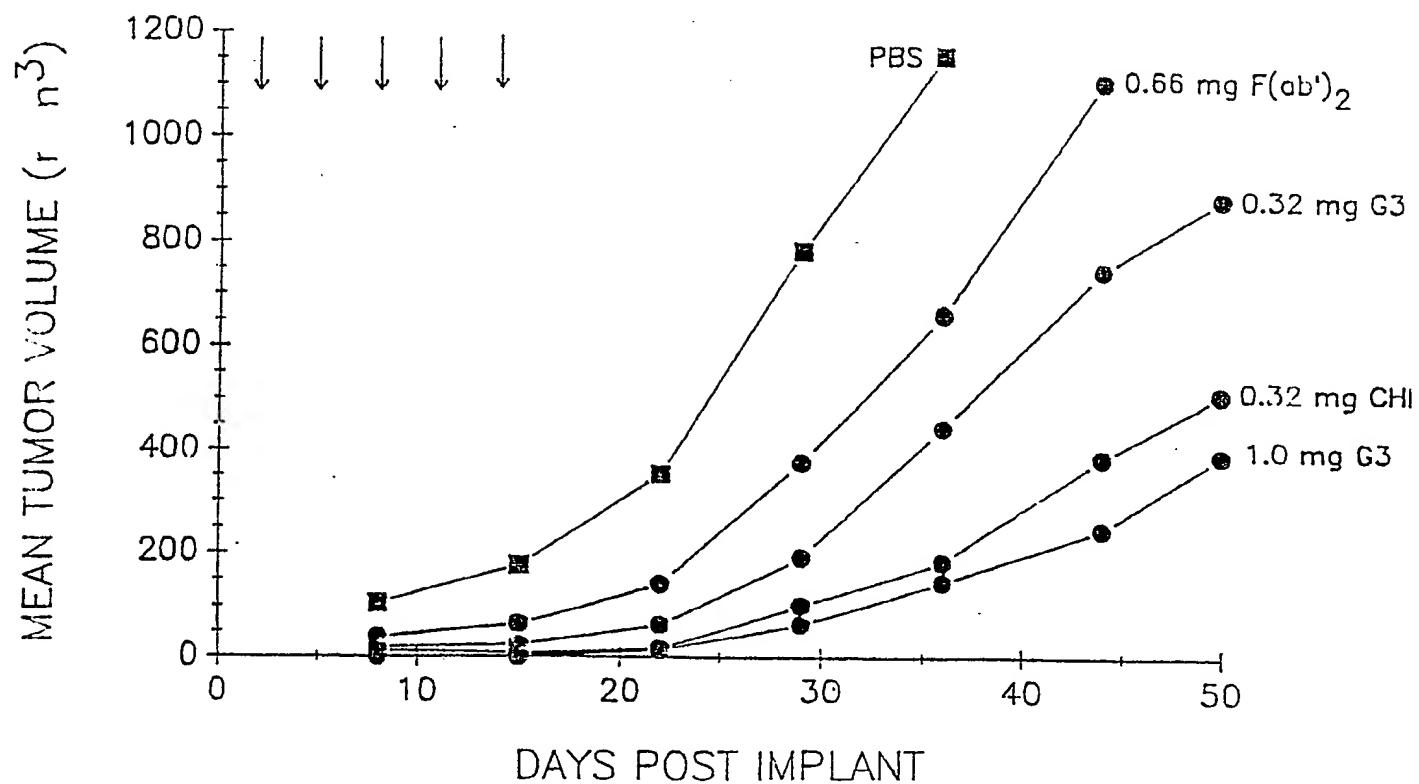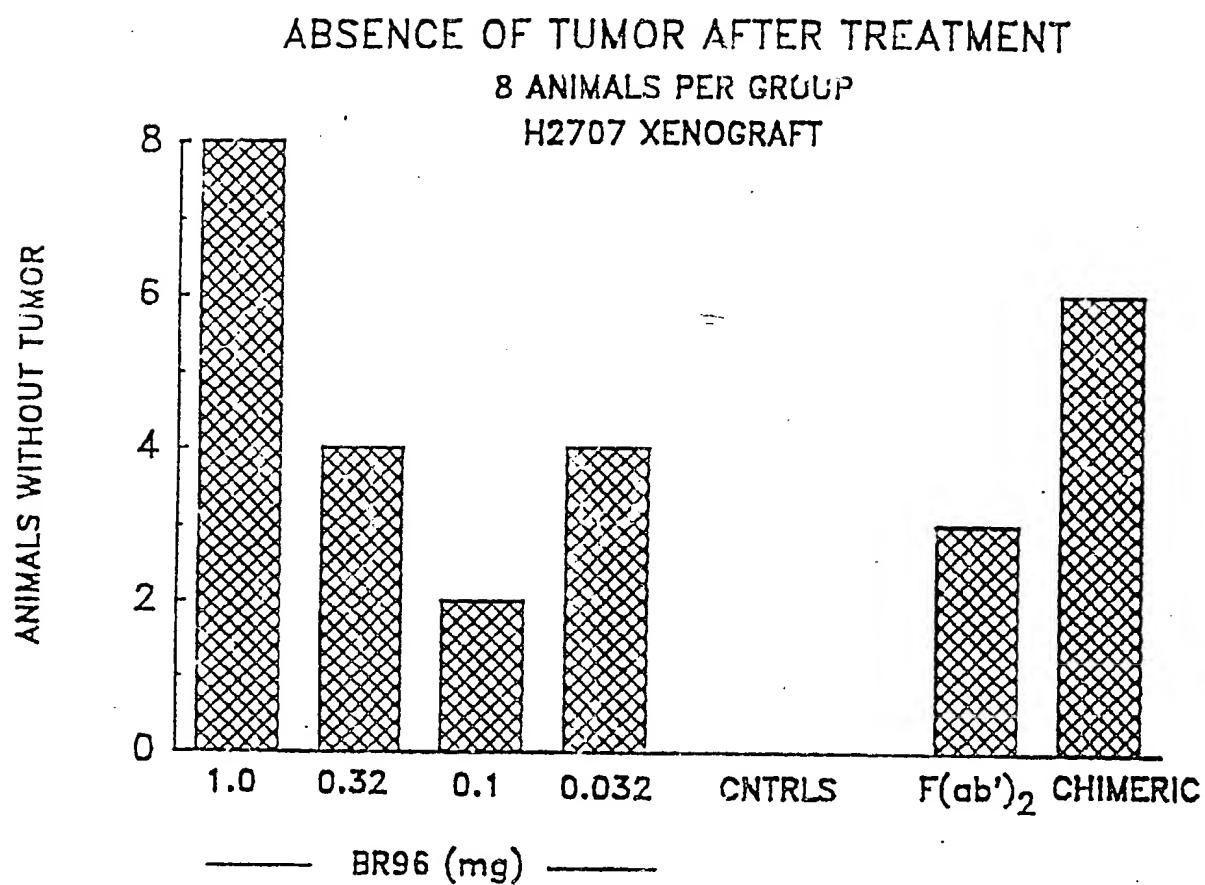
FIGURE 27

International Application No. PCT/US90/03712

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C07K 15/28; C12N 5/12, 9/96; COIN 33/535; A61K 43/00 , 39/00
US. CL.: 530/387,388,389; 435/240.27,188,7; 424/1.1,85.8,85.91

II. FIELDS SEARCHED

Minimum Documentation Searched
Classification System

Classification Symbols

U.S.	530/387,388,389; 424/1.1,85.8,85.91; 435/240.27, 188,7.

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched

DATABASES: STNINTERNATIONAL ONLINE (FILE CA, 1967-1990; FILE BIOSIS, 1969-1990) DIALOG ONLINE (FILE BIOSIS; FILE MEDLINE; FILE CANCERLIT) AUTOMATED PATENT SYSTEM (FILE US PAT. 1971-1990)

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category [*]	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
X Y	Bioscience Reports, Volume 3 issued 1983, A. Brown et al., "A monoclonal antibody against human colonic adenoma recognizes difucosylated Type-2-blood-group chains", pages 163-170, see page 163.	1-4,15,16 5-14,17-31
X Y	Cancer Research, volume 46, issued November 1986, Y.S. Kim et al. "Expression of Le^y and extended Le^y blood group-related antigens in human malignant, premalignant and monmalignant colonic tissues", pages 5985-5992, see page 5985.	1-4,15,16 5-14,17-31
X Y — —	Cancer Research, volume 46, issued May 1986, K. Abe et al. "Differential expression of difucosyl type 2 chain (Le^y) defined by monoclonal antibody AH6 in different locations of colonic epithelia, various histological types of colonic polyps and adenocarcinomas", pages 2639-2644, see page 2639.	1-4,15,16 5-14,17-31

* Special categories of cited documents: [15]
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [‡]	Date of Mailing of this International Search Report [‡]
12 September 1990	9 NOV 1990

International Searching Authority [‡]	Signature of Authorized Officer [20]
ISA/US	Paula Hutzell

III. DOCUMENTS CONSIDERED TO BE RELEVANT   (CONTINUED FROM THE SECOND SHEET)

Category*	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	Monoclonal Antibodies '84: Biological and Clinical Applications, published 1985, P.E. Thorpe, "Antibody Carriers of Cytotoxic agents in Cancer Therapy: a Review", see pages 475-506.	9-11,18-20,22, 26,30.31
Y	Goding "Monoclonal Antibodies: Principles and Practice" published 1983, see pages 118-125.	12
Y	WO, A, 89/05309 (Kohler et al.) 15 June 1989, see entire document.	29
Y	Science, Vol. 229, issued 05 July 1985, M. Brennan et al. "Preparation of bispecific antibodies by chemical recombination of monoclonal immunoglobulin G_1 fragments, pages 81-83, see especially page 83.	13,14
Y	Journal of Radiation Oncol. Biol. Phys., Vol. 13, No. 10, issued October 1987, L. Brady et al., "Therapeutic and diagnostic uses of modified monoclonal antibodies, pages 1535-1544, see entire document.	21-29
Y	Science, Vol. 229, issued 20 September 1985 S. Morrison, "Transfectomas Provide Novel Chimeric antibodies, pages 1202-1207, see entire document.	5-11,17-19

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET		
X Y	Immunogenetics, volume 17, issued 1983, K.O. Lloyd et al. "Mouse monoclonal antibody F3 recognizes the difucosyl type-2 blood group structure", pages 537-541, see pages 537-538.	1-4,15,16 5-14,17-31
X Y	International Journal of Cancer, volume 41, issued 1988, J. Bara et al. "Ectopic expression of the Y(Le^Y) antigen defined by monoclonal antibody 12-4LE in distal colonic adenocaranomas", pages 683-689, see page 683.	1-4,15,16 5-14,17-31

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers ...... because they relate to subject matter [1] not required to be searched by this Authority, namely:

2. ☐ Claim numbers.......... , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [1], specifically:

3. ☐ Claim numbers_____ , because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows: Group I, claims 1-4, 12, 15, 16, 21/3 and 24/3 to antibodies, fragments, hybridomas, pharmaceutical compositions, method of treatment; Group II claims 5,6-8,11, 17,20/7,21/7,24/7 to recombinant protein, chimeric antibody, hybridoma, pharmaceutical compositions; Group III, claims 9,10,23 to protein conjugate, pharmaceutical composition; Group IV, claims 13, 14 to bispecific SEE ATTACHMENT

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.